The Effects of a Single Bout of Plyometric Exercise on Anabolic and Catabolic Osteokines in Girls and Adolescents

By

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Abstract

It is well established that dynamic mechanical loading is both beneficial and necessary to the promotion and development of healthy bones. The aim of this study is to determine the response of osteokines related to the anabolic Wnt signalling pathway [sclerostin and dickkopf 1 (DKK-1)] and the catabolic RANKL pathway, [osteoprotogerin (OPG), receptor activator of nuclear factor kappa-β ligand (RANKL)], as well as the related transforming growth factors (TGF-β1, TGF-β2 and TGF-β3) to an acute bout of plyometric exercise in girls and adolescent females. Twenty six females, 14 girls (10.5 ± 0.4 years of age) and 12 adolescents (15.0 ± 0.3 years of age) were recruited to participate in this study. Serum samples were collected pre, 5 min post, 1 hour post and 24 h post exercise. Group differences were seen at baseline in DKK-1, TGF-β1, TGF-β2 and TGF-β3 with girls having significantly higher concentrations than the adolescents. A significant decrease was found after 24 hours in DKK-1. A significant decrease was also found in RANKL at 5 minutes post exercise that remained suppressed 1 hour and 24 hours following the cessation of the exercise protocol in both groups. Plyometric exercise was therefore successful in suppressing the catabolic osteokines DKK-1 and RANKL up to 24 hours following the cessation of exercise in girls and adolescent females.

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Abbreviations

ANOVA	Analysis of variance
BAP	Bone alkaline phosphatase
BIA	Bioelectrical impedence analysis
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CTx	C-telopeptides of Type I collagen
%CV	Coefficient of variation
DPP-4	Dipeptidyl peptidase-4
Dsh	Dishevelled receptor
ELISA	Enzyme-linked immunosorbent assay
FFQ	Food Frequency Questionnaire
FSH	Follicule stimulating hormone
Fzd	Frizzled receptor
GLP-1	Glucagon-like peptide-1
GSK-3β	Glycogen synthase kinase-3β
IGF-1	Insulin-like Growth Factor 1
LRP	Low density lipoprotein
LRP5	Low density lipoprotein receptor 5
LRP6	Low density lipoprotein receptor 6
NTx	N-telopeptides of Type I collagen
OC	Osteocalcin
OPG	Osteoprotogerin

PHV	Peak height velocity
P1NP	N-Terminal Propeptides of Type I Collagen
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-β ligand
RM ANOVA	Repeated measures analysis of variance
SGLT-2	Sodium glusoce-linked transporter-2
TGF-β	Transforming growth factor-β
TRAP5b	Tartrate-resistant acid phosphatase 5b
WA_{eq}	Weekly physical activity metabolic equivalent

Chapter 1: Introduction

1.1 Rationale

Physical activity is an important factor of bone health, particularly in children. Wolff's Law states that "the form or function of a bone is followed by adaptive changes in its internal architecture and its external shape" (1); in a pediatric population, physical activity is an important stimulus for this adaptive change. The response of bone to physical activity can be measured in a variety of ways. When studying physical activity in the acute sense (over hours); common measures are bone biomarkers. These can be in the form of direct measures of bone formation or resorption (termed bone turnover markers) or indirect indicators of formation or resorption activity (termed osteokines). Mechanical loading through physical activity has been shown to influence a variety of osteokines in human adult populations. Sclerostin, which is an inhibitor of the anabolic Wnt signalling pathway, has been proposed to have a negative linear relationship with physical activity (2). Similar to sclerostin, dicckopf-1 (or DKK-1) is an inhibitor of the anabolic Wnt signalling pathway. Its response to exercise is unknown. Conversely, the catabolic nuclear factor kappa-β ligand (RANKL) pathway is also affected by physical activity with RANKL, and its decoy receptor osteoprotegerin (OPG) being stimulated through physical activity (3). However, pediatric populations are largely understudied when it comes to these anabolic and catabolic osteokines. Therefore, the aim of this research is to address this gap in the literature. A window of insight into the effects of physical activity is seen in cross-sectional studies examining different types of sports on bone properties in children. Children who participate in high impact sports such as artistic gymnastics show greater bone mineral density (BMD) than swimmers or recreationally active children (4).

The amount of physical activity undertaken daily is also a significant predictor of bone health in pediatric populations (5) as well as adult male populations (6).

Physical activity and its relationship with bone parameters (such as biochemical markers of bone turnover) are typically studied in three ways: acutely, in the short term (0-6 months), or over a long term (6 months or longer). Typically, short term and long term studies are undertaken to determine the effects of an exercise intervention on makers of bone turnover independent of bone growth through the use of an intervention group and a control group. In children, these interventions tend to be school-based and involve plyometric protocols such as jumping, as this type of plyometric training has been shown to produce sufficient forces to stimulate a bone response (7,8). Less often, however, are the acute effects of a single exercise session examined in relation to markers of bone turnover. Even rarer are pediatric studies of the acute response of bone parameters to exercise. Physical activity is key to achieving an optimal peak bone mass in late adolescence and early adulthood; yet very little research has been done to investigate difference types of exercise and their potential to alter bone metabolism. To date, only two studies have examined the effects of acute exercise on bone markers in children. One examined cycling in pubertal boys – a mode of exercise that is not considered osteogenic – and only measured a few markers of bone turnover (9). The other compared the effects of a single session of plyometric training on bone turnover markers in boys and men. They demonstrated elevated markers of both formation and resorption in boys but not men 24 hours following the exercise session (10) and that sclerostin responded to exercise in men but not boys (11).

1.2 Objectives and Hypothesis

The overall purpose of this study was to determine the response of osteokines related to the anabolic Wnt signalling [sclerostin and dickkopf 1 (DKK-1)] and the catabolic RANKL pathways, [osteoprotegerin (OPG), receptor activator of nuclear factor kappa-β ligand (RANKL)], as well as the related transforming growth factors (TGF-β1, TGF-β2 and TGF-β3) to an acute bout of plyometric exercise in girls and adolescent females. The specific objectives of this study are: (1) To compare the resting serum levels of anabolic and catabolic osteokines as well as transforming growth factors between girls and adolescents, (2) to determine the acute effects of a single bout of plyometric exercise on other markers/osteokines and growth factors associated with the Wnt and RANKL pathways over 24 hours in girls and adolescents, and (3) to determine whether there are differences in these acute responses between the two groups. A secondary aim of this research is to determine whether energy expenditure and dietary intake play a role in these responses to a single bout of plyometric exercise.

It is hypothesized that: (1) resting serum levels of osteokines and growth factors will be higher in the girls than the adolescents, (2) osteokines and growth factors will increase one hour after exercise and will return to baseline after 24 hours, and (3) girls will demonstrate higher levels of all markers than adolescents but both groups will show the same pattern of variation in response to the acute protocol.

Dynamic mechanical loading (such as plyometric exercise) promotes bone formation in both children and adults. When the rate of bone turnover is balanced in equal rates of formation and resorption, bone mineral density is maintained. When this rate is shifted in favour of bone formation, bone mineral density is increased; when shifted in

favour if resorption, bone mineral density is decreased. However, the acute anabolic and catabolic response of bone metabolism to this plyometric exercise is not fully understood. Since acute exercise is capable of stimulating shifts in bone metabolism, it can help us better understand the relationship between bone metabolism and exercise. A better understanding of the relationship between bone metabolism and exercise is most certainly beneficial, if not to aid in achieving optimal peak bone mass, then certainly to aid in preventing age-related bone diseases such as osteoporosis. Since the literature on this topic is limited in adults and scarce in children, this study will aid in the standardization of timing of blood sampling in order to study bone metabolism as well as enable a direct comparison of bone metabolism in children and adults.

Chapter 2: Review of Literature

2.1 General Bone Physiology

2.1.1 Bone Anatomy, Development and Turnover

Bone can be divided into two components: cortical bone and trabecular bone (also known as cancellous bone). Cortical bone is heavily mineralized to provide structural support, and is located in the diaphysis or shaft of the long bones (12,13). Trabecular bone is calcified to a lesser extent than cortical bone and is found at the epiphyses of long bones. Trabecular bone has a greater surface area than cortical bone, which allows it to be more metabolically active (12,13).

Structurally, there are two type of matrices within bone; the organic matrix which is comprised of mostly Type I collagen as well as the inorganic or mineral matrix which is comprised of mostly calcium and phosphate (1,12,13).

Within the organic matrix, three types of cells predominate: osteoblasts, osteoclasts and osteocytes. Osteoblasts are bone-forming cells that synthesize and mineralize the organic matrix. Osteoclasts are bone-resorbing cells that locally degrade bone matrix (12). Osteocytes are derived from osteoblasts trapped within the bone matrix (12) and account for 90-95% of adult bone cells. Among other possible functions, these cells respond to mechanical strain via signals of formation and resorption (14). When mechanically loaded, osteocytes recruit osteoblasts to form new bone. The bone remodelling cycle begins when osteoclasts on the bone surface are activated; typically in response to osteocyte apoptosis and begin to resorb the surface bone matrix (1). The deficit left in the bone is then filled by osteoblasts. This entire process takes approximately 200 days to complete in adult populations (13) (see Figure 1).

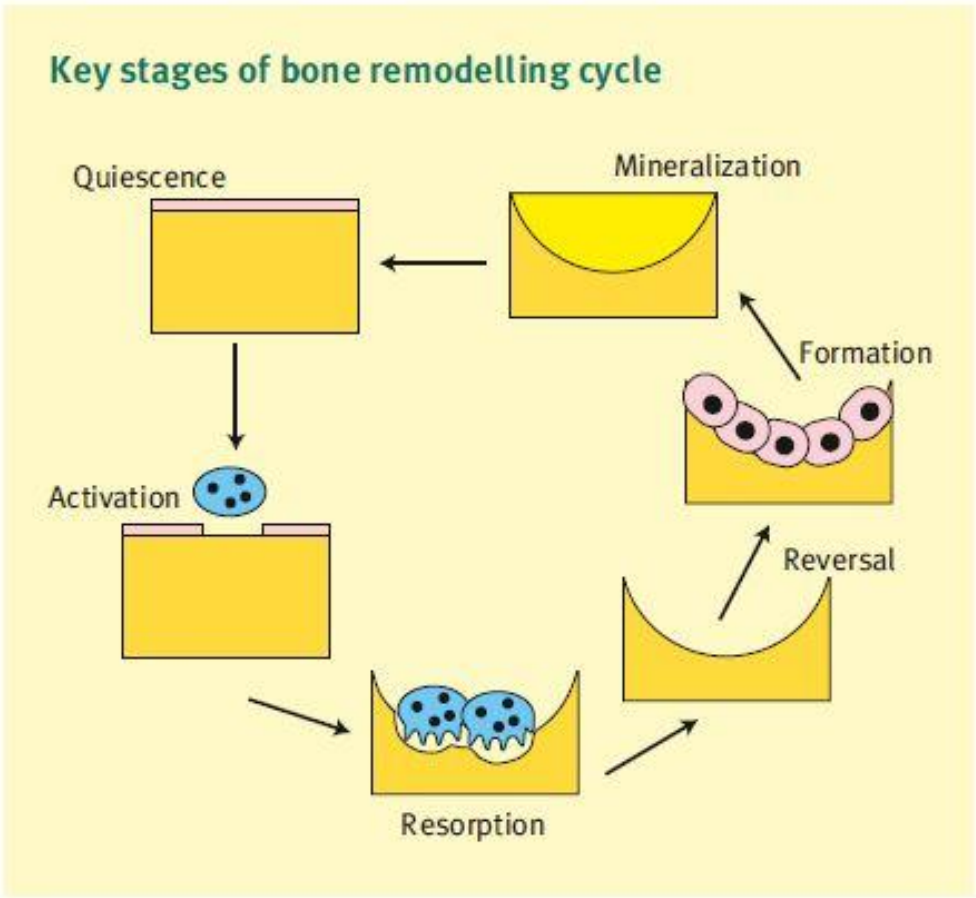

Figure 1. Bone Cells and Bone Turnover. Osteoclasts resorb bone and the bone defect is built back slightly stronger than before by the osteoblasts (13).

2.1.2 Wnt Pathway

The Wnt/β-catenin signalling pathway - also referred to as the canonical Wnt pathway or the canonical β-catenin-dependent Wnt signalling pathway is involved in determining cell fate, proliferation and survival (15). Wnt signalling is a bone anabolic pathway and is an important component of the induction of osteoblastogenesis. Wnt signalling promotes the renewal of stem cells, stimulates preosteoblast replication, osteoblast differentiation as well as inhibits osteoblast and osteocyte apoptosis (16).

Wnts are cysteine-rich secreted glycoproteins that interact with the co-receptor complexes low-density lipoprotein receptor 5 (LRP5), low-density lipoprotein receptor 6 (LRP6) and the frizzled receptor (Fzd) (14,15,17–20). This interaction causes the activation of the dishevelled receptor (Dsh) via phosphorylation (14,17) which in turn results in the phosphorylation of glycogen synthase kinase-3β (GSK-3β). This causes the phosphorylation of β-catenin which accumulates and translocates to the nucleus, where it begins to affect gene transcription (14,15,17–19) in favour of bone formation (14–17) (see Figure 2).

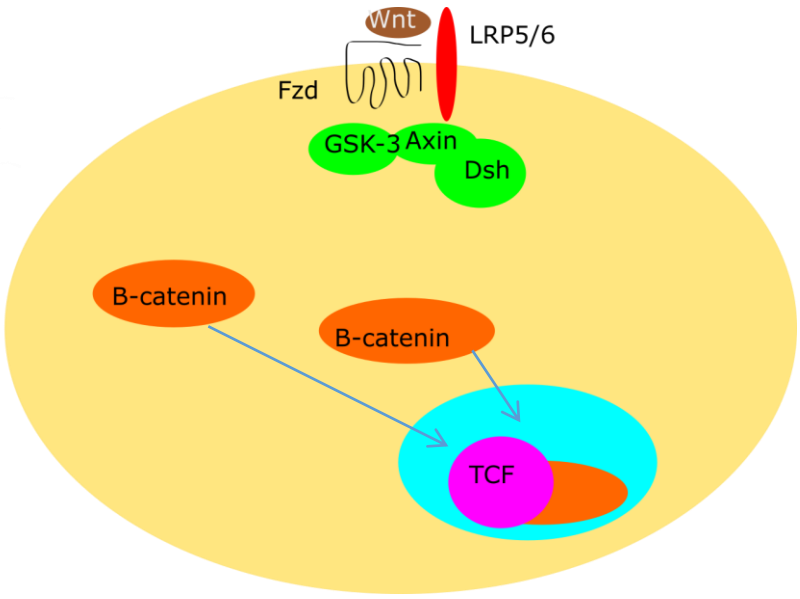

Figure 2. Wnt Signalling. Cysteine-rich secreted glycoproteins **Wnts** attach to the low-density lipoprotein receptor complex 5 and 6 **(LRP5 and LRP6)** and the frizzled receptor (**Fzd**). This causes the activation of **Dsh** and **GSK-3β** which in turn causes **β-catenin** to accumulate. This β-catenin then translocates to the nucleus to affect gene transcription (TCF) in favour of bone formation (17).

2.2 Biochemical Factors Associated with the Wnt Pathway

2.2.1 Sclerostin

The discovery of the protein sclerostin and the identification of its role in bone catabolism and turnover resulted in an increased interest in the mechanism by which it interacts with other markers of bone metabolism. Sclerostin is an atypical, soluble (21) cysteine knot secreted glycoprotein that is expressed by the Sost gene in osteocytes (18,19,21–23). Sclerostin has been shown to be secreted primarily within osteocytes (19,21,23–29) and to some extent within chondrocytes (26). Osteocytes secreting sclerostin are more likely to be buried deep within the bone matrix, rather than at the superficial bone surface (21,26–28).

Sclerostin works as an osteokine to inhibit bone formation through antagonizing the canonical Wnt signalling pathway (19,21,22,24,25,27,30–32). By inhibiting the actions of LRP5 (2,18,19,21,22,24–27,32,33) and LRP6 (18,19,22,25,27,32,33), sclerostin inhibits osteoblastogenesis by preventing the formation of the active LRP complex (33) which inhibits the Wnt pathway from allowing bone formation to occur. Thus, sclerostin indirectly promotes osteoclast function (34). Sclerostin has demonstrated catabolic effects on bone within the animal model in the sense that targeted reductions in sclerostin levels lead to increases in bone mass (33).

Sclerostin is secreted by osteocytes after they become imbedded in the mineralized matrix, though the specific mechanism is still unclear (25). This indicates that high levels of circulating sclerostin can cause even greater bone loss as osteoclasts formed in this environment are more effective at resorbing the bone matrix than pre-

existing osteoclasts. In an animal model it has been shown that high rates of bone formation are present in the absence of sclerostin (35). In human disease states where sclerostin is low (such as in menopause), it has been shown that serum sclerostin levels are significantly higher in post-menopausal women than their pre-menopausal counterparts (36–42). In human disease states, rare gene mutations can lead to sclerosteosis or van Buchem disease; both characterized by an inability of the osteocyte to transcribe Sost in order to express sclerostin. Both of these diseases present with enlargement of facial and mandibular bones and can cause cranial nerve damage and is associated with sensory loss (25). Animal models have demonstrated that overexpression of sclerostin leads to decreases in BMD of the axial skeleton through the suppression of the Wnt pathway as well as blunting the osteogenic response to mechanical loading (43). The suppression of sclerostin has been shown to induce bone formation even in the absence of prior resorption (32). Through animal models, it has also been shown that targeted sclerostin deletion leads to a high accrual of bone mass (33). It has therefore been hypothesized that sclerostin antibodies could aid in the reduction of the negative effects of sclerostin on bone formation. Early trials have indicated that antibody-based sclerostin inhibition increases bone mass through increased bone formation coupled with decreased bone resorption (26,33,44). This antibody-based treatment has been suggested as a new method of treating bone loss such as the age-related bone loss seen in osteoporosis (21).

2.2.2 DKK-1

DKK-1 is a glycoprotein secreted primarily by osteoblasts and osteocytes (45) that attaches to the LRP 5/6 receptor complex (46,47) to directly inhibit Wnt signalling (45), directly contribute to bone loss (48) as well as suppress bone formation (49), much like

its counterpart sclerostin (46). DKK-1 is therefore another osteokine that directly inhibits osteoblast-mediated bone mineralization (45) by preventing osteoblastogenesis and promoting programmed cell death (46). In the animal model, expression of DKK-1 is limited to bone in adult animals (45), though it can be expressed in endothelial cells elsewhere, such as in the cardiovascular system (50). DKK-1 has also been linked to osteoclast development *in vivo* within the animal model (51).

DKK-1inhibits Wnt-dependent osteoblast differentiation (51,52) as well as Wnt-induced production of OPG (51) and subsequently increases the expression of RANKL (52). Overexpression of DKK-1 has been demonstrated to lead to osteopenia (45), however, neither DKK-1 nor sclerostin have been linked to fracture risk in older adults (53). Serum levels of DKK-1 may also be involved in glucocorticoid- and estrogen deficiency- mediated osteoporosis (46), and negatively associated with bone mineral density (52). Serum levels of DKK-1 are higher in post-menopausal women who are osteoporotic compared to those who are not osteoporotic (54).

In the animal models, anti-DKK-1 antibodies have also been shown to have a dose-dependent decrease in osteoclast count (48) as wells as a significant increase in new bone formation (49). Decreased serum DKK-1 concentrations due to antibody treatment are also correlated with increased markers of bone formation such as P1NP and OC as well as being correlated to positive measures of bone strength such as increased bone mass, improved bone microstructure and decreased bone loss (55). Pharmaceutical therapies such as biophosphates are also successful in decreasing DKK-1 levels in post-menopausal osteoporotic women (54).

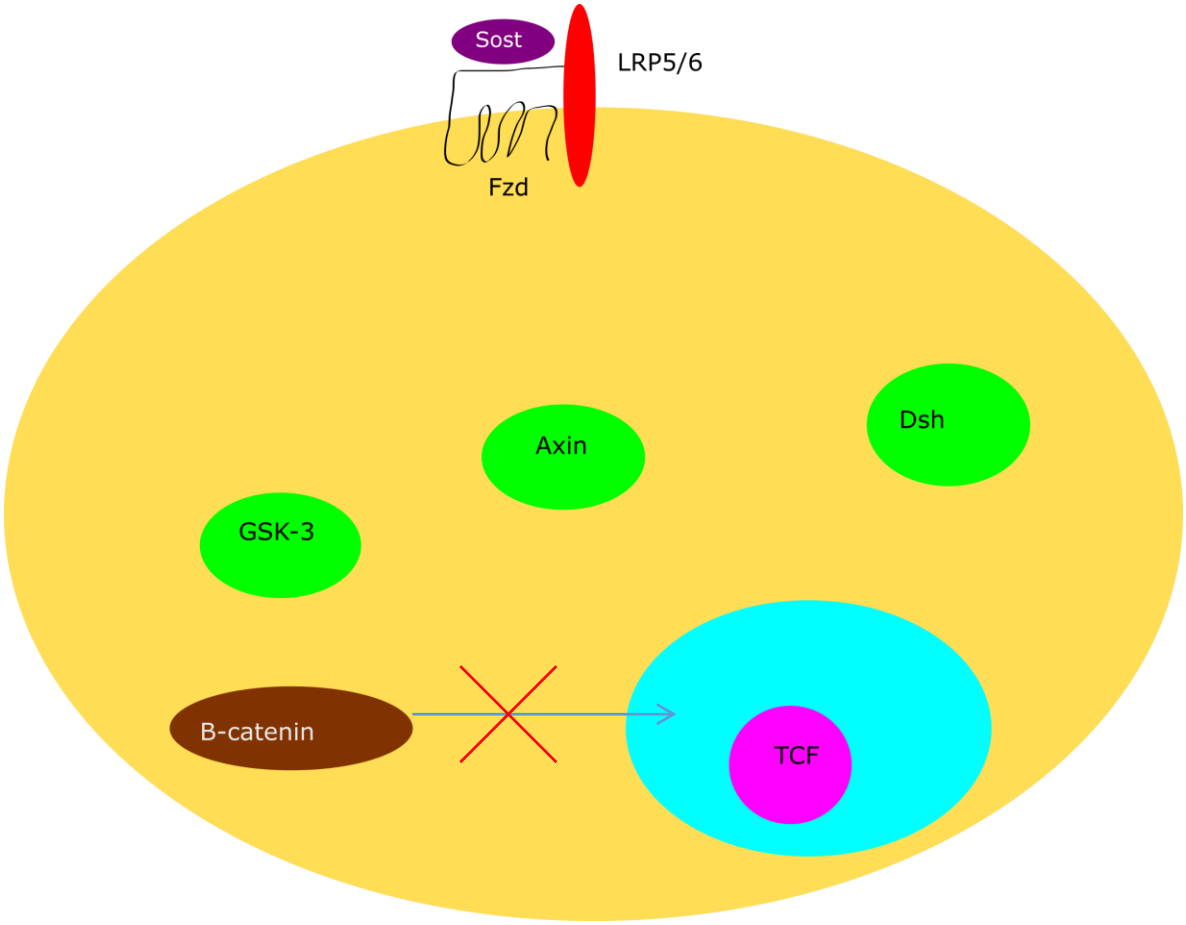

Figure 3. The Wnt Pathway When sclerostin **(Sost)** and dicckopf 1 **(DKK-1)** attach to LRP5/6, the entire cascade that allows for gene transcriptions that promote bone formation to occur is blocked (26).

2.2.3 TGF-β

The Transforming growth factor-β (TGF-β) is expressed in cells of the immune system as well as in osteoblasts, is also involved in osteoclastogenesis along with osteoclast regeneration (56) and osteoblasts have high concentrations of TGF-β receptors (57). There are three different isoforms of TGF-β: TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is found largely in superficial, transitional or immature bone cells and is the first to

respond to bone fracture through either secretion by platelets or synthesis from osteocytes (57). The isoforms of TGF-β1 and TGF-β2 are structurally very similar, however their concentration and actions are quite different. TGF-β1 concentration in bone is 4 times greater than that of TGF-β2; yet the action of TGF-β2 is 3.75-fold higher than that of TGF-β1 (57). TGF-β2 responds more transiently than TGF-β1 and is present once TGF-β1 have decreased while TGF-β3 concentrations and activity remain largely stable over time (57), although the greatest effect of TGF-β3 on bone formation is seen in a longer time frame (20-30 days) (58).

TGF-β can influence bone formation independently of the Wnt pathway, and/or it can interact with the Wnt pathway (TGF-β1 in particular) to block DKK-1 (59), and therefore, can prevent inhibition of Wnt signalling. Independent from the Wnt pathway, TGF-β2 has been shown to induce the corticalization and mineralization of bone tissue (60). TGF-β3 appears to be the most direct contributor to bone formation as it is capable of inducing significant osteogenesis through the upregulation of osteocalcin (or OC, a potent bone formation marker), as wells as influencing the differentiation of stem cells into an osteoblastic lineage (61). Aside from bone formation during growth or healing from fracture, all TGF-β isoforms found in mammals are involved in the ossification of non-bone tissues (62), in particular TGF-β1 (63) and TGF-β2 (62).

It has also been suggested that TGF-β can directly influence the Wnt signalling pathway by preventing phosphate-induced transcription factors, essential to bone formation, from translocating to the nucleus of the osteocyte – namely β-catenin (64). When interacting with the Wnt pathway, TGF-β is released from osteoclasts and activated during bone resorption (56) and works to increase bone resorption through

altering the OPG:RANKL ratio (65). TGF-β is another marker of the anabolic response to bone and it has be demonstrated that the repression of TGF-β via mechanical loading is another important regulator of sclerostin (66). The presence of TGF-β induces the expression of sclerostin (67,68) and exerts its effects on bone metabolism independently of PTH-mediated pathways (67); however, this effect may be greater in the animal model than in the human model, as TGF-β has been found to have negligible effects on sclerostin expression in human cell lines (69) when compared to animal cell lines. The animal model also suggests that increased concentrations of TGF-β in turn result in increased concentrations of DKK-1 (64).

2.2.4 OPG/RANKL

Nuclear factor kappa-β ligand (RANKL) is secreted by many tissues including the osteoclast and is a marker of bone resorption that is necessary for osteoclast function (32,70) through promoting complete differentiation of osteoclast precursors into mature osteocytes (71) as well as the induction of osteoclast activation (72). When RANKL binds to its associated receptor RANK on osteoclast precursors, it allows osteoclast differentiation and function to be enhanced (73,74). RANKL stimulates the fusion of osteoclasts to bone and subsequently promotes their activation and survival (70,74) as well as promoting osteocyte apoptosis (32).

Osteoprotegerin (OPG) is a decoy for RANKL (32,70,73,74) that is secreted by osteoblasts (73,74) to prevent osteoclast differentiation and activity (71) and accelerate osteoclast apoptosis (70,72) namely through preventing the actions of nuclear factor kappa-β ligand (RANKL) (71,72). Increases in levels of OPG have been associated with decreased osteoclast number as well as increased bone strength and bone density in the

animal model (70). Uninhibited Wnt signalling stimulates the production and secretion of OPG, thereby antagonizing RANKL and decreasing osteoclast differentiation (20).

The ratio of OPG to RANKL is a critical regulator of osteoclast function and bone resorption (73). RANKL is a marker of bone resorption. Although increased levels of RANKL are associated with increased bone resorption (32,70), the presence of RANKL alone is insufficient to stimulate resorption independently (70). In order for resorption to be mediated by RANKL, there needs to be an associated decrease in OPG (70).

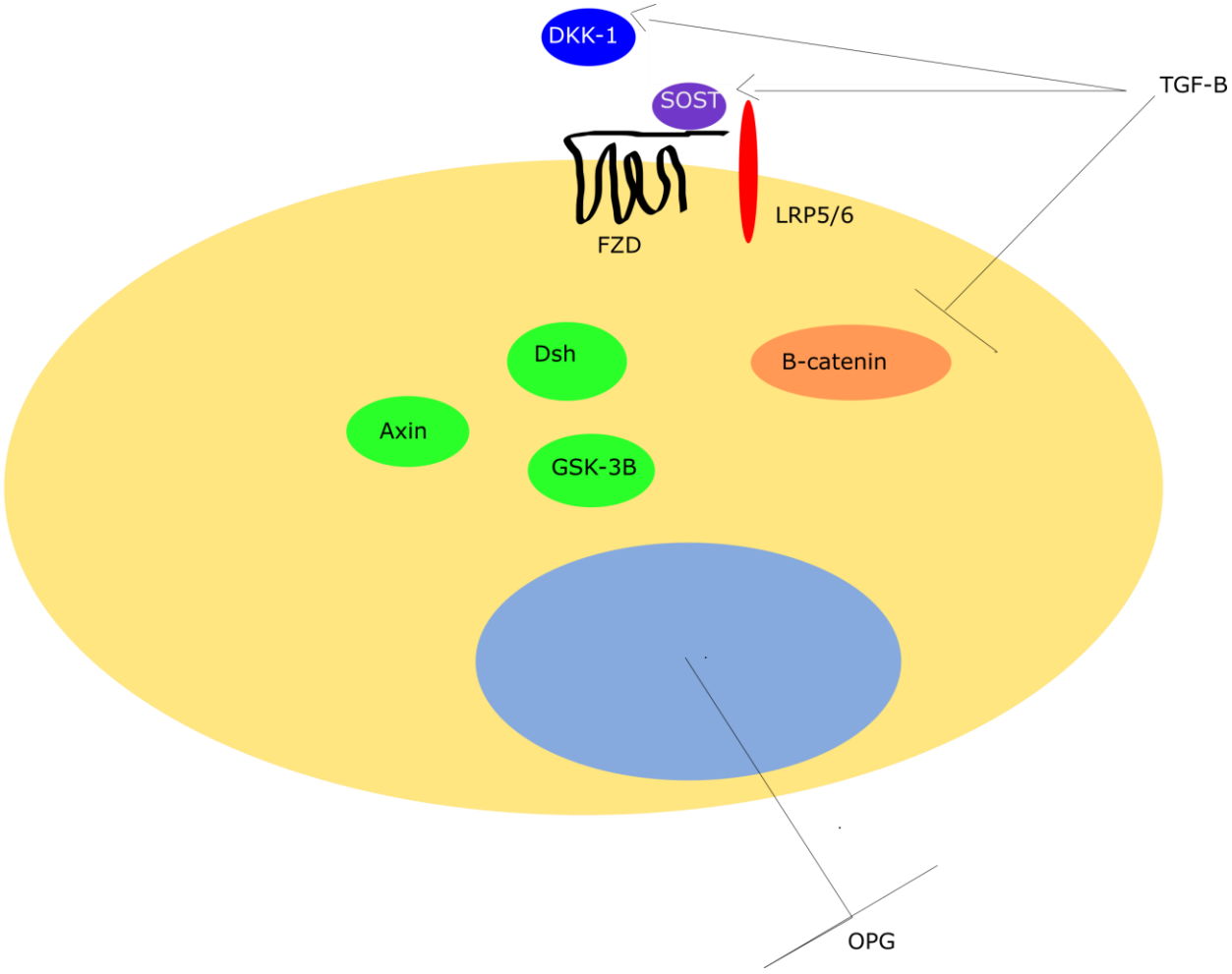

Figure 4. Osteokines and Growth Factors Related to the Wnt Pathway. Both sclerostin and DKK-1 inhibit the Wnt signalling pathway in the osteocyte to prevent β-catenin from translocating to the nucleus, decreasing the transcription of markers such as OPG, leading to an increase in RANKL. TGF-β directly prevents β-catenin from translocating to the nucleus as well as increasing transcription of sclerostin and DKK-1 to inhibit the Wnt pathway.

2.2.5 Across the Lifespan

2.2.5.1 Children and Adolescents

The role of osteokines such as sclerostin and DKK-1 in human bone development in athletic and non-athletic pediatric populations has been largely understudied. Early

investigations indicate that serum sclerostin is higher in pre-pubertal boys than pre-pubertal girls (75), and higher in pediatric weight-bearing athletes compared with non-athletes (76). In a healthy pediatric population, whether recreationally active or weight-bearing athletes, there appears to be no linear relationship between classical bone parameters such as bone turnover or BMD and serum sclerostin (75,76). However, the presence of a "split point" or non-linear relationship between sclerostin and chronological age has been postulated. It has been shown that before the age of 10 years in girls and 14 years in boys, there is a positive association between sclerostin and bone age, and after this split point, there is a negative association between sclerostin and bone age (75). This indicates the potential of a maturational effect of sclerostin levels, and therefore could offer a window of opportunity to promote bone development.

When it comes to levels of OPG and RANKL in children, it would appear there is also no relationship between either marker, nor the ratio of the two, on BMD in adolescent females (77). When approaching sexual maturity, serum concentrations of OPG steadily increase, and continue to increase after sexual maturity, whereas RANKL steadily decreases while approaching sexual maturity and continues to decrease after sexual maturity has been reached (77). There is no difference in levels of RANKL between obese and normal weight children, however serum levels of OPG in obese children are decreased compared to their normal weight counterparts. OPG is also correlated with trunk and total fat mass in children whereas RANKL is not (78).

Neither TGF-β nor DKK-1 have been studied in healthy pediatric populations. DKK-1 is not associated with total fat mass in either obese children or normal weight controls (78). TGF-β is largely studied in disease states, such as Marfan Syndrome (79).

Our study will be one of the first to address this gap in the literature. Since literature regarding these two markers in healthy children is limited, this research can aid in the knowledge of health bone development as well as potentially play a role in preventing disease states later in life.

2.2.5.2 Adulthood

Sclerostin has been shown to be higher in adult men than adult women regardless of age (18). Serum sclerostin levels are significantly lower in pre-menopausal women aged 30-34 than 35-39, and appears to be stable across the ages of 35-45 (80). In pre-menopausal women, those who accumulated greater than 120 minutes of physical activity in a week showed significantly lower serum sclerostin levels than their inactive counterparts (81). Serum sclerostin levels display no association with BMD in pre-menopausal women (82). Furthermore, there are no racial differences in sclerostin levels in pre- or post-menopausal women (42).

The majority of research into the effects of sclerostin on human bone has involved older adults, particularly older women. Serum sclerostin levels are significantly higher in post-menopausal women than their pre-menopausal counterparts (36–42), though this has largely been shown in women not receiving estrogen therapies including oral contraceptives (37). In post-menopausal women, sclerostin was found to be negatively correlated to whole body BMD and BMC even when adjusted for age and BMI (81). In addition, sclerostin has been found to be elevated in female osteoporotic populations when compared to female non-osteoporotic populations (70), therefore, serum sclerostin has also been suggested as a predictor for fracture risk in post-menopausal women (33,81,83).

The literature concerning DKK-1 in human adults is scarce. DKK-1 is significantly higher in osteoporotic males and females compared to osteopenic males and females (54), and is also significantly higher in post-menopausal women who are osteoporotic compared to those who are not (84). DKK-1 is also inversely correlated to bone mineral density in older female populations (54). However, limited research exists in this area and therefore, the mechanism by which DKK-1 is involved in aging is not well understood.

In older adult populations, OPG is significantly higher in older women than older men, while no differences were seen in serum levels of RANKL (85). When comparing osteoporotic post-menopausal women to their non-osteoporotic counterparts, RANKL is significantly higher (86), however there appears to be no difference between these two groups in OPG concentrations (84). There also appears to be an increase in RANKL levels post-menopause when compared to pre-menopausal women and this relationship has been suggested to be due to the estrogen deficiency seen after menopause (70,74). Estrogen deficiency in menopause has also been suggested as the mechanism by which OPG levels are seen to decrease after menopause (74). Together, this net increase in RANKL:OPG could contribute to age-related bone loss.

There is also limited evidence regarding the levels of and changes in TGF-β during adulthood. The research that is available appears to focus on TGF-β1 and its role in bone loss later in life. It has been shown that TGF-β1 is significantly higher in early menopause than late and pre-menopause (with no differences between late and pre) (87), and that in late menopausal women, there are no differences in TGF-β1 levels between individuals who are osteoporotic and those who are not (88). TGF-β1 is also not

associated with hip or total body BMD in older women and a significant linear positive relationship exists between TGF-β1 levels and fracture risk in older women, while a non-linear negative relationship appears to exist between TGF-β1 levels and fracture risk in older men (89), posing a potentially interesting sex difference in TGF-β1 levels in older adults.

2.3 Exercise and Bone

It has been suggested that there are several factors that affect the clinical efficacy of exercise on bone remodelling, these being age, sex, diet and drugs, exercise intensity and type as well as time course of measurement and the presence of metabolic disorders (90). The following sections aim to address these factors and their effects on bone metabolism.

2.3.1 Exercise and Bone Mineral Density

Bone matrix micro-damage, which can be accrued through physical activity, stimulates remodelling and increases in bone strength. Physical activity is known to improve bone micro-architecture as well as influence bone geometry (1).

Differences in bone accrual between participants of different sport types or between athletes and non-athletes in pediatric populations mainly appear after puberty (4,5,91,92). Additionally, there is a lack of consensus as to whether peak strain is a better indicator of increasing BMD than total physical activity (6).The Iowa Bone Development Study, a longitudinal bone health study that is currently ongoing, has suggested that a significant predictor of hip BMC is the amount of vigorous physical activity performed before the age of five in girls aged 13 and 15 (93).

In adolescent populations, there is less of a consensus than there is in adults on the effects of exercise on bone turnover. A recent review suggests that cumulative daily physical activity is not enough to explain differences in bone mass in female adolescents (6). Femoral neck BMD is only affected if physical activity in adolescence is carried over into adulthood and increases in lumbar spine bone mass is only associated with physical activity done in adulthood. In children, this relationship has again been shown to be largely sport or activity specific although the literature on exercise and bone turnover in younger children is very limited.

In pediatric populations, cross sectional studies can give us a glimpse of the effects of exercise on BMD. Gymnastics is a high impact sport and those who participate in these sports have increased femoral neck BMD compared to other athletes exposed to extreme dynamic loading and/or weight bearing activity such as endurance running (94). This provides evidence that perhaps the repetitive dynamic loading of gymnastics is beneficial in the development of bone strength. However, there are differences in BMD across gymnastic specialties. Artistic gymnasts have higher BMD compared with rhythmic gymnasts, swimmers and age-matched non-athletic controls (76). Rhythmic gymnasts only demonstrate differences from controls and swimmers at the femoral neck (4), suggesting the higher dynamic loads of artistic gymnastics are required to see differences in total body BMD. Soccer and tennis, which dynamically load the lower and upper limbs respectively, demonstrate increased BMD compared to swimming and non-athletic controls (91). Synchronized swimming, a low-impact sport due to its aquatic nature, is shown to have no significant differences on bone speed of sound (an indicator of bone strength and therefore BMD) compared to age-matched controls (95).

Exercise intervention studies provide a better design to examine the effects of exercise on BMD. According to the systematic review conducted by Ludwa and Klentrou, (96), which included 35 exercise intervention studies, short-duration, high-impact exercise interventions undertaken early in childhood (pre and early puberty) have demonstrated a persistent long-term effect on BMD and BMC over and beyond that of normal growth and development. A recent meta-analysis by Ishikawa et al. (2013) examined the impact of weight-bearing exercise on areal bone mineral density (aBMD) and BMC in young girls with the objective to quantify the influence of key moderating variables (e.g. pubertal stage, exercise mode, intervention strategy, exercise duration, frequency of exercise, program length and study design) on skeletal development. The authors analyzed 17 exercise training studies and concluded that interventions performed for more than 3 days per week and lasted for at least one year were more effective in promoting bone development in growing females (97). The meta-analysis by Behringer et al. (2014), which included 27 intervention studies, showed a significant effect of weight-bearing activities on BMC only in pre-pubertal children suggesting that the efficacy of training in terms of bone mineral accrual is substantially affected by the maturational status of participants(98). Finally, the most recent systematic review published by Specker et al. (2015) included 22 exercise interventions and in consistence with the previous reviews, they also showed benefits on aBMD in pre-pubertal children but not in children who were early or post-pubertal (99).

2.3.2 Exercise and Bone Turnover Markers

The successful promotion of bone development via exercise is largely movement-specific. Evidence in adults suggests that bone formation markers are extremely sensitive

to even minor changes in physical activity, such as the transition from performing no daily physical activity to adding walking to one's daily routine (100). The majority of research on the relationship between exercise and bone turnover markers has not focused on the markers that will be investigated in this study, therefore the discussion that follows will concern more traditional bone turnover markers in the literature such as osteocalcin (OC) bone alkaline phosphatase (BAP), cross-linked C telopeptide and cross-linked N-telopeptide (CTx and NTx respectively), tartrate-resistant acid phosphatase 5b (TRAP5b) and N-terminal type I pro-collagen (P1NP).

2.3.2a Short Term Training

The effects of short term training offers activity-specific insights into its relationship with markers of bone turnover (101). Small changes in bone turnover markers can be seen in just two weeks with decreases in TRAP5b as well as decreases in BAP while OC remains unchanged (102). A review found that five to eight weeks of endurance running appears to favour bone formation by decreasing bone resorption markers (101). Different training modalities appear to favour different changes in bone turnover markers, with 8 weeks of endurance training having minimal changes in markers of either formation or resorption while resistance training significantly increased BAP and OC following eight weeks of training. A combined aerobic and resistance training protocol increased BAP following eight weeks of training with increases in OC only being demonstrated at the midpoint of training (103). Ten weeks of military training (a combination of both aerobic and anaerobic exercise) in adult females saw increases in the formation markers BAP and PINP as well as increases the resorption markers CTx and TRAP (104). This indicates that the combination of anaerobic and aerobic exercise modes

led to a net increase in bone turnover. The type of resistance training appears to have an effect as well, as ten weeks of upper body resistance training alone showed no change in either markers of formation or resorption (105). Anaerobic and resistance training appears to have the overall effect of increasing bone formation by either favouring bone formation with no change or decreases in bone resorption (101).

2.3.2b Acute Exercise

The most responsive marker of bone turnover to acute exercise appears to be OC though the effects typically are determined by the type and duration of the exercise (106). In an acute session of plyometric training in untrained males, OC has been shown to be elevated five minutes and one hour following the cessation of exercise with similar elevations demonstrated in an eccentric protocol two hours, one day, three days and even five days after a high volume eccentric workload (107). Treadmill running has been shown to increase OC levels during exercise though concentrations of OC returned to baseline levels at the end of the exercise. OC then increased and recovered during a recovery period, with no changes at follow-up days (108) indicating differences in the response of OC to different types of exercise. Jogging has demonstrated no effect on OC levels in young women, though a response was seen in post-menopausal women (106).

The effects of an acute session of exercise on BAP appears to be activity specific. Aerobic weight bearing sessions of jogging or treadmill running appear to be the mode to demonstrate changes in serum BAP with walking decreasing serum BAP 24 hours post-exercise in young women (109). BAP has also been seen to increase immediately after jogging in post-menopausal women then returning to baseline after 24 hours, again confirming the specificity of activity needed to demonstrate elevations of BAP (106). The

addition of resistance exercise to a walking protocol increases serum BAP 24-hours post-exercise (109). Resistance training appears to independently increase BAP only immediately after exercise, with markers of bone resorption significantly lowered one and eight hours following an exercise session, and no change in P1NP (110). Eccentric exercise (whether high or low volume) appears to have no effect on serum BAP both immediately or on follow-up days (107).

P1NP is one of the few measures studied acutely in children. In boys, it is unchanged after a single exercise bout (9). An acute session of weight bearing exercise (treadmill running) has been shown to increase P1NP significantly in adult males during exercise wherein it decreases rapidly and significantly during the recovery period of four days (108).

2.3.3 Exercise and Factors Related to the Wnt Pathway

The main function of the osteocyte is largely mechanosensory (21), and mechanical loading is one of the most convincing regulators of sclerostin expression by Sost (18). Mechanical loading in the animal model has been shown to decrease sclerostin expression by the Sost gene resulting in increased bone formation (2,21,22,24–26,32,111).The local distribution of osteocytes producing sclerostin can control the formation of new bone whether or not bone remodelling will take place, indicating that the relationship between sclerostin and bone remodelling has a site-specific mechanism (21,25,26,111). Several animal trials have indicated that joints experiencing high peak strain when mechanically loaded show higher reductions in sclerostin levels within the osteocytes compared to joints exposed to lower peak strains when mechanically loaded

(2,111). A linear relationship between amount of local mechanical loading and the subsequent local sclerostin response has also been postulated (2).

The research regarding the relationship between exercise and DKK-1 is scarce. It has been reported that in adults, resistive exercise may have an effect on DKK-1, but the study was too small to detect any significant changes (112) and therefore the effects of exercise on DKK-1 are relatively unknown.

The literature regarding the relationship between TGF-β and exercise is scarce. However, one study has shown that TGF-β appears to be responsive to strenuous physical activity. Evidence in young adult males performing strenuous cycling (a mode of exercise that is of a high intensity but low mechanical load) indicates that TGF-β increases significantly immediately after a bout of strenuous exercise and remains elevated two hours after the cessation of the exercise bout (113).

Exercise (or the lack of) is capable of increasing levels of RANKL within the osteocyte (32). In post-menopausal women performing 8 months of either resistance or aerobic weight bearing exercise, no changes are seen in levels of OPG, RANKL or in the ratio between the two markers (114). Another study showed that 32 weeks of combination of resistance and balance training in older adults significantly increased levels of OPG in women but not men, with no changes in RANKL in either group (85). Exercise that is of low impact but also of high intensity is capable of increasing OPG and RANKL 5 min after an exercise session, but both markers return to baseline within 24 hours in healthy, recreationally active males (3). A recent study determined that an acute session of plyometric exercise significantly increased BAP and OPG in both boys and men, but that

the time course of this increase was different between the two age groups; whereas RANKL was not significantly different between boys and men, nor was it different after exercise. It was therefore postulated that an acute session of plyometric exercise was sufficient to stimulate bone formation in both boys and young men (115).

2.4 Growth and Development

2.4.1 Role of Biological Sex on Growth and Development

Biological sex is an important uncontrollable factor when it comes to bone mass and bone turnover. Cortical BMD is significantly greater in adolescent and adult females when compared to males of the same age with medium to large effect sizes for the female sex on bone mass-related variables (116). A medium-large effect size for the male sex is present for rates of bone formation and resorption. Adolescent males have higher concentrations of bone turnover markers (both formation and resorption) thus leading to an increased rate of bone turnover (116). Markers of bone formation also peak at different ages between males and females. Serum bone biomarkers peak at Tanner stage II in girls and Tanner stage III in boys (117). BAP peaks around the age of 9 in girls and 12 in boys, while OC peaks at 12 in girls and 13 in boys, with significant decreases in the serum levels of these markers following peak levels (118). However, it has also been suggested that bone mass is only associated with bone turnover markers in Tanner stage I, suggesting biological sex is not the only factor affecting growth and bone turnover (117).

The relationship between bone turnover and menstrual phase is important to consider in females. Estrogen increases the sensitivity of bone to mechanical loading (119) and also appears to be the main factor influencing BMD and BMC in both pre-pubertal boys and girls (120). Early in the follicular phase, bone turnover markers such

as CTx and OPG are suppressed. In the luteal phase and during ovulation, these resorption markers decrease, following an inverse relationship with estrogen fluctuations during difference phases (119,121). The differences in estrogen between pre-menarche and post-menarche girls can be accommodated by measuring post-menarche girls during the follicular phase when estrogen is low (122).

2.4.2 Controllable Factors

Pharmacological agents for conditions other than osteoporosis and other bone disorders can influence BMD and bone health. Anti-hyperglycemic medications use to treat diabetes can have differing effects. Metformin has been postulated to increase BMD and therefore decrease fracture risk whereas GLP-1 and DPP-4 are thought to have no impact on bone health (123). Treatments such as insulin, thiazolidinediones, and SGLT-2 have been shown to increase fracture risk either by increasing hypotension (SGLT-2) or decreasing BMD (thiazolidinediones). (123). Finally, anti-epileptic medications have demonstrated convincing osteopenic effects, with both enzyme-inducing antiepileptic medications and non-enzyme-inducing antiepileptics showing decreases in BMD (124).

Oral contraceptive use also plays a role in bone turnover as well as BMD. Use of oral contraceptives containing estrogen have shown significant decreases in markers of bone turnover (125,126), however spinal BMD appears to remain unchanged, even after six (127) or twelve (128) months of use. Exercise may provide a protective effect against this decrease in bone turnover (125) and oral contraceptive use may be protective against losses in bone strength associated with eating restraint (129). Long term use of oral contraceptives containing estrogen may also decrease BMD by allowing an inadequate amount of bone mineral accrual (125). Research concerning oral contraceptive containing

only progestin provide no clear evidence for their role in bone turnover, though it is speculated they may increase levels of bone resorption markers (125).

Calcium and Vitamin D are the most common dietary components that can affect BMD. Unfortunately, a large population study found that only 17% of Canadian children aged 9-13 are meeting the adequate intake for calcium (130). Calcium is of particular importance in the stages of early puberty as bone mineral absorption and deposition rates peak just before menarche (131). Mild pediatric Vitamin D deficiency is detrimental to bone mineral accrual (131) and is also important as Vitamin D needs to be present in the body in order to see the full benefits of adequate dietary calcium (132), such as optimal growth and increased BMD. Increasing calcium consumption through diet and/or supplementation has been shown to potentially have greater longer term effects when taken earlier in puberty than later (131).

Lastly, as previously mentioned, physical activity is a strong controllable factor in promoting bone turnover and formation, particularly in the formative years. It would appear that a window of opportunity exists in the pre-pubertal stages for girls, where greater increases in BMC at the femoral neck and lumbar spine have been seen with impact exercise compared to their mid-pubertal counterparts (8,133). Jumping exercise has been shown to increase hip BMD in both boys and girls (134) and longitudinal studies have indicated that long term physical activity contributes to greater increases in BMC in both boys and girls (135,136) with the greatest effects of physical activity being seen at weight bearing sites such as the tibia and femoral neck (136).

Chapter 3: Methods

3.1 Participants

This study and all related procedures received ethical clearance from the Brock University Biosciences Research Ethics Board (REB 14-267 KLENTROU). Twenty-six females (14 girls and 12 adolescents) completed the study. Participants were recruited through poster advertisements, newspaper ads and word of mouth. Recruitment of participants was focused on recreationally active girls and adolescents to limit the effects of elite or high volume sport participation on markers of bone turnover. Exclusion criteria included factors known to influence bone turnover properties: (1) BMI $\geq$ 90% percentile for their age, (2) previous (within last 6 months) or current fracture, and (3), pharmaceutical use of agents which may affect bone.

3.2 Procedures

All testing took place at the Applied Exercise Physiology Laboratory of Brock University. Participants were asked to refrain from consuming caffeine and alcohol for 6 hours prior as well as refraining from vigorous or high impact exercise for a minimum of 24 hours prior to testing. The first visit began between 8 and 9 am for all participants in order to control for circadian rhythm variation. Upon arrival, participant and parents/guardians were familiarized with the purpose of the study, the measurements involved, and any potential risks or benefits. The informed consent form was signed by the parent/guardian and the informed assent form signed by the participant.

After obtaining consent and assent, the resting blood sample was drawn using a standard venipuncture technique. Anthropometric measures of height, seated height and weight (kg), as well as body composition were performed. A questionnaire package

including medical history, Godin-Shephard Leisure Time Exercise Questionnaire, Food Frequency Questionnaire and pubertal Stage questionnaire were completed by all participants, with parental/guardian help if needed. The plyometric exercise protocol was then completed and followed by two post-exercise blood samples, 5 min post-exercise and 60 min post exercise. During the second visit, the last blood sample (24 hours post-exercise) was taken.

3.3 Exercise Protocol

The exercise protocol has been designed to provide high-impact, weight bearing loads in the form of circuit training stations. By following this protocol, a minimum of 100 jumps were executed. This protocol was previously used in our lab by Kish and colleagues (10) that was adapted from MacKelvie and colleagues in their longitudinal school-based interventions (8) that has been successful in eliciting a bone response in pediatric populations.

The participants began the session with a warm-up that included 5 min of low intensity cycling. Once the warm-up was completed, participants were given a comprehensive explanation and demonstration of each of the exercises. Following this explanation, the participants were allowed to familiarize themselves with the exercises to control for learning effects and to avoid injury.

Each participant was instructed to rotate through each of the five stations (Jumping jacks, lunge jumps, hopping, tuck jumps and drop jumps) three times for a total of three sets. Jump height was tailored to each participant based on body size. The participant was at each station for approximately two minutes before she was instructed to

move to the next station. There was a rest period of three minutes between each set. Previous work has demonstrated that the ground reaction forces involved in such as circuit typically involves three to five times body weight (7) and is, therefore, a sufficient stimulus to induce a detectable bone response.

3.4 Measurements

3.4.1 Anthropometry and Body Composition

Height and seated height were measured to the nearest 0.1 cm using a stadiometer, with no shoes and light clothes. Body mass (kg) as well as body composition including lean body mass (LBM), fat mass (FM) and percent body fat (%BF) were measured using the InBody520 bioelectrical impedance analysis (BIA) system (Biospace.228). Body mass index was calculated by dividing the participant mass in kilograms by their height squared in metres. Waist to hip ratio is calculated by dividing the circumference of narrowest point measured at the waist (in cm) by the widest point of the circumference of the hips (also in cm). The same investigator performed all measures of height (cm), seated height (cm), weight (kg) and body composition for all participants.

3.4.2 Indicators of Maturity

Sexual maturity was self-assessed using secondary sexual characteristics (pubic hair and breast development) according to Tanner Staging (137). Somatic maturity was assessed by calculating years from age of peak height velocity (PHV), using height, seated height, leg length, as well as body mass (138).

3.4.3 Physical Activity Measures

Leisure time physical activity was assessed using the Godin-Shephard Leisure Time Exercise Questionnaire. This questionnaire evaluates leisure time physical activity using indicators of the intensity of exertion as light, moderate or strenuous (139). From the answers provided by the participants, weekly physical activity metabolic equivalent (WA_{eq}) can be assessed based on the number of 15 min blocks at each intensity level. The number of blocks at each intensity level is then converted to metabolic equivalents (139). The Godin-Shephard Leisure Time Exercise Questionnaire has been demonstrated to be valid and reliable in male and female pediatric, adolescent as well as adult populations (140).

3.4.4 Dietary Intake Measures

Dietary intake was evaluated using a food frequency questionnaire. Specifically, the Block Questionnaire for Children is designed to assess dietary habits through a recall of foods eaten in the last week and has shown to be a valid nutritional assessment in a pediatric population (141). Pictures are provided of portion sizes to enhance accuracy. The adolescent participants used the Adult Block Questionnaire 2014. All responses were analyzed by NutritionQuest (NutritionQuest, USA) providing estimates of total energy intake (kcal/day).

3.4.5 Biochemical Markers

Venous blood samples were collected from the antecubital fossa of each participant using a standard venipuncture technique between 8:00 am and 9:00 am to avoid circadian rhythm variations in bone turnover markers. The first sample (pre-exercise) contained eighteen millilitres of blood and all remaining samples contained ten

millilitres of blood (post-exercise samples). Serum was collected using SST vacutainers. One pre-exercise, and three post-exercise blood samples were collected at 5 min, 60 min and 24 hours following the exercise bout. The blood was allowed to clot for 30 min at room temperature before being centrifuged for 15 min at 4˚C at 1000 x gravity. The serum was then separated and aliquoted into 1.5ml polyethylene tubes which were then stored at -80°C until analysis. To test for potential post exercise hemoconcetration, pre and post exercise hematocrit measurements were performed in 6 participants and no changes in plasma volume were observed.

Serum samples were analyzed for biochemical markers. The following osteokines were measured using Milliplex MAP kits: sclerostin, DKK-1, OPG and RANKL. The following growth factors were also analyzed using Milliplex MAP kits: TGF-β1,2 and 3. The use of Milliplex kits allows for efficient use of time, as several markers can be measured on the same plate (Human Bone plate containing sclerostin, DKK-1 and OPG as well as a RANKL plate and a TGF-β plate containing TGF-β1,2 and 3). Since these kits are capable of detecting pediatric concentrations of these markers, serum was diluted according to kit-specific instructions.

All samples and kits were brought to room temperature prior to the initiation of the analysis procedure. All analyses were performed in triplicate due to the nature of a Milliplex kit and concentrations were read using a MAGPIX ® xPONENT ® plate reader.

Sclerostin, DKK-1 and OPG were assayed using 5 Milliplex ® MAP Human Bone Magnetic Bead Panel kits (EMD Millipore Corporation, Billerica, MA, USA). The

procedure of this assay is split over two days. On the first day, all reagents necessary were brought to room temperature and reconstituted. When completed, the plate was then sealed with a plate sealer, wrapped in foil and left to incubate for 16-18 hours on a plate shaker at 4˚C. The following morning, all remaining reagents were removed from the fridge and allowed to warm to room temperature. The plate was then read using xPONENT ® software for the MAGPIX ®. The intra-assay coefficients of variability for sclerostin were 1.86%, 11.96%, *23.34*%, 0.65%, 1.14% and 23.75%, respectively. The inter-assay coefficient of variation for sclerostin was 9.0 %. The intra-assay coefficients of variation for DKK-1 were 0.16%, 1.75%, 13.24%, 5.03% and 0.18%, respectively. The inter-assay coefficient of variation for DKK-1 was 18.5%. The intra-assay coefficients of variation for OPG were 0.09%, 0.44%, 1.01%, 2.13% and 21.44%, respectively. The inter-assay coefficient of variation for OPG was 12.1%.

Nuclear factor kappa B ligand (RANKL) was assayed using 5 Milliplex ® MAP Human Bone Magnetic Bead Panel kits (EMD Millipore Corporation, Billerica, MA, USA). The procedure of this assay is split over two days. On the first day, all reagents necessary were brought to room temperature and reconstituted. When completed, the plate was then sealed with a plate sealer, wrapped in foil and was left to incubate at 4˚C for 16-20 hours with agitation. The following morning, all remaining reagents were removed from the fridge and allowed to warm to room temperature. The plate was then read using xPONENT ® software for the MAGPIX ®. The intra-assay coefficients of variation were 0.89%, 4.6%, 2.9%, 1.11% and 12.3%, respectively. The inter-assay coefficient of variation was 12.8%.

Transforming Growth Factor β1, 2 and 3 was assayed using 5 Milliplex ® MAP TGFβ 1,2,3 Magnetic Bead kits (EMD Millipore Corporation, Billerica, MA, USA). The procedure of this assay is split over two days. On the first day, all reagents necessary were brought to room temperature and reconstituted. When completed, the plate was then sealed with a plate sealer, wrapped in foil and was left to incubate at 4˚C for 16-18 hours with agitation. The following morning, all remaining reagents were removed from the fridge and allowed to warm to room temperature. The plate was then read using xPONENT ® software for the MAGPIX ®. The intra-assay coefficients of variability of TGF-β1 were 0.41%, 0.99%, 1.25%, 6.9% and 5.14% respectively. The inter-assay coefficient of variation was 18.3%. The intra-assay coefficients of variation for TGF-β2 were 1.08%, 0.62%, 0.62%, 5.82%, and 0.35%, respectively. The inter-assay coefficient of variation was 19.1%. The intra-assay coefficients of variation for TGF-β3 were 0.59%, 1.23%, 0.09%, 4.09% and 4.35%, respectively. The inter-assay coefficient of variation was 19.9%.

3.5 Statistical Analysis

All variables were normally distributed and no outliers were present in the data. Independent *t*-tests were used to examine differences between groups in anthropometric measures, habitual physical activity, nutrition intake as well as baseline measures of bone markers. A two-way repeated measures analysis of variance (RM ANOVA) with group as the between-subject effect and time the within subject effect was used to assess group differences over time for sclerostin and other osteokines. Post hoc analysis was used if significant main effects were detected. For each group, mean values at each time point were used for analysis. Missing values (n = 12 of 56 for the girls, n = 6 of 48 for the

adolescents) were accounted for using the group's mean value for that particular time point. Missing time points were created if a blood sample was unable to be taken at a particular time point. If the assumption of sphericity for a particular case is not met, the Greenhouse-Geisser test of significance was used. If the assumption of equality of covariance is not met, than Pillia's Trace test of significance was used. Data are reported as means and standard errors. Significance was assumed at an alpha level of 0.05. The observed power of this study was 0.850. Statistical analysis was performed using SPSS version 22.0 for Windows.

Chapter 4: Results

Participants' physical characteristics are presented in Table 1. As expected, the girls were significantly younger, shorter and had a lower percent body fat than the adolescents. There were also significant differences in measures of somatic maturity (age from peak height velocity) and sexual maturity (Tanner stage), with the adolescents significantly further along in their growth and development. There were no significant differences in waist to hip ratio (a measure of the distribution of adiposity). Tanner Stage was unavailable for one girl in the adolescent group.

Table 1. Physical characteristics of girls and adolescents.

	Girls (n=14)	**Adolescents (n=12)**	***P***
Age (years)	10.5 ± 1.8	15.0 ± 1.0	<0.0001*
Height (cm)	145.7 ± 11.2	163.3 ± 7.7	<0.0001*
Weight (kg)	37.5 ± 9.4	59.9 ± 8.4	<0.0001*
Body Fat (%)	16.9 ± 6.0	25.4 ± 8.1	0.0100*
Age from PHV (years)	-1.1 ± 1.5	2.4 ± 0.7	<0.0001*
Waist to Hip Ratio (cm)	0.7 ± 0.0	0.8 ±0.0	0.8310
Pubertal stage **(n in stage 1,2,3,4,5)**	8,2,4,0,0	0,0,3,3,5	

All values are presented as mean ± standard deviation
*indicates a significant difference between groups

Table 2. Habitual physical activity, dietary calcium, dietary Vitamin D and total energy intake of girls and adolescents.

	Girls (*n*= 14)	**Adolescents (n=12)**	***P***
METs per week	100.14 ± 36.5	92.4 ± 34.7	0.606
Calcium (mg/d)	592.33 ± 199.7	1348.71 ± 706.7	0.001*
Vitamin D (IU/day)	110.7 ± 61.9	254.3 ± 168.4	0.007*
Total Energy Intake (kcal/d)	1150.67 ± 284.1	2208.39 ± 1079.7	0.002*

All values are presented as mean ± standard deviation
*indicates a significant difference between groups

There were no differences between groups in leisure time physical activity as assessed by the Godin Shephard Leisure Time Exercise Questionnaire (Table 2). All participants were recreationally active and none were competitive athletes in high impact sports such as gymnastics. In addition, there was no correlation between reported leisure time physical activity and baseline levels of osteokines or growth factors. Significant differences between groups are present in daily calcium intake, daily vitamin D intake as well as total daily energy intake; in all cases the girls had lower intakes than the adolescents group (Table 2).

Table 3. Resting serum levels of osteokines and transforming growth factors (TGF-β) in girls and adolescents.

Biochemical Marker	Girls	Adolescents	*p* value
Sclerostin (pmol/L)	31.52 ± 6.2	32.53 ± 3.4	0.618
DKK-1 (pmol/L)	29.76 ± 6.5	23.36 ± 4.8	0.010*
OPG (pmol/L)	10.70 ± 5.1	8.41 ± 3.3	0.196
RANKL (pmol/L)	2.48 ± 0.4	2.47 ± 0.7	0.956
TGF-β1 (ng/ml)	0.238 ± 0.06	0.164 ± 0.08	0.015*
TGF-β2 (ng/ml)	0.022 ± 0.0	0.016 ± 0.0	0.002*
TGF-β3 (ng/ml)	0.0005 ± 0.0	0.0004 ± 0.0	<0.0001*

All values are presented as mean ± standard deviation
*indicates a significant difference between groups using t-test.

At baseline, there were no group differences in sclerostin, OPG and RANKL (Table 3). Significant differences were detected in DKK-1, TGF-β1, TGF-β2 and TGF-β3 with the girls displaying higher levels of these markers in all four instances than the adolescents (Table 3).

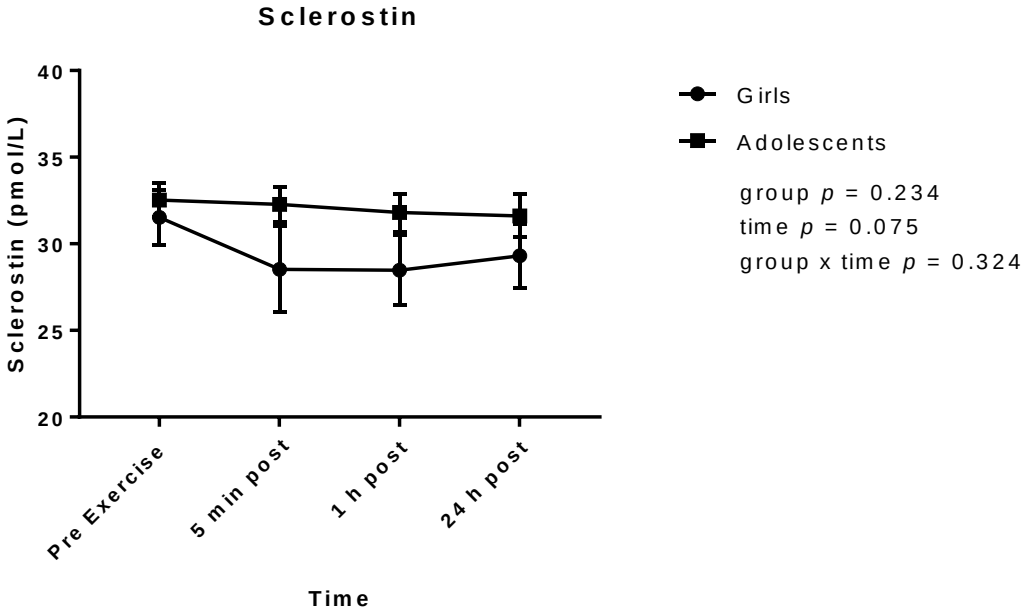

Figure 5. Sclerostin response to the plyometric exercise in girls and adolescents. All values are presented as mean ± standard error.

There was no significant interaction (p>0.05) and no significant group effect for sclerostin (p>0.05) (Figure 5). There was a trend towards a main time effect (p=0.075). The observed power for sclerostin was 0.761.

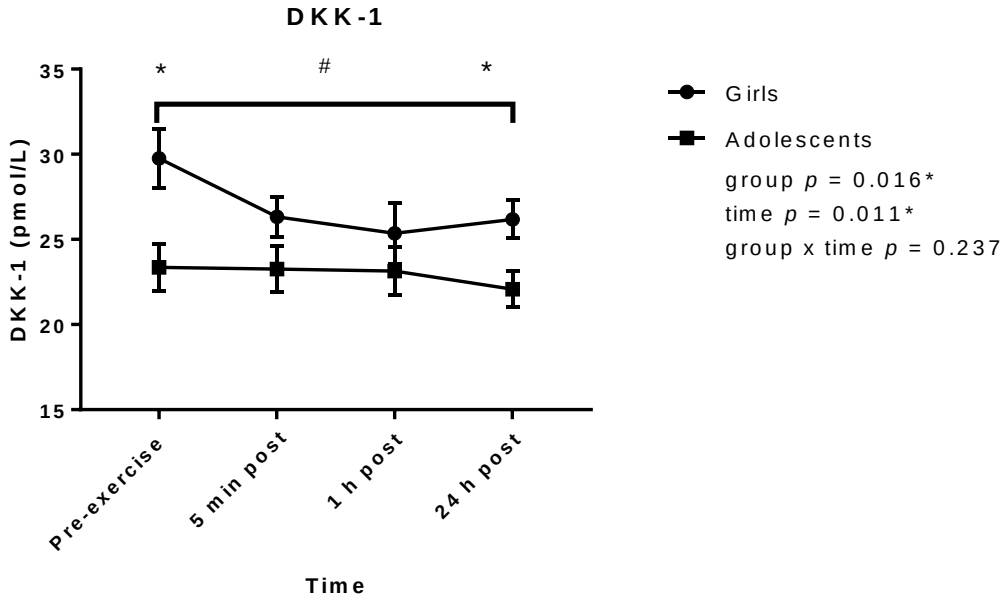

Figure 6. DKK-1 response to the plyometric exercise in girls and adolescents.
All values are presented as mean ± standard error;
* indicates a significant difference between groups
# indicates a significant difference between time points

There was no significant interaction (p>0.05) for DKK-1. A significant group effect (p=0.016) was present for DKK-1. Post hoc analysis revealed a significant difference between groups pre exercise (p=0.010), as also shown in the t-test analysis, as well as 24 hours post exercise (p=0.015) with girls displaying significantly higher levels of DKK-1 than adolescents. There was also a significant effect for time (p=0.011) with a significant decrease at 24 hours post-exercise compared to baseline levels of DKK-1 (p=0.013) in both groups (Figure 6). The observed power for DKK-1 was 0.756.

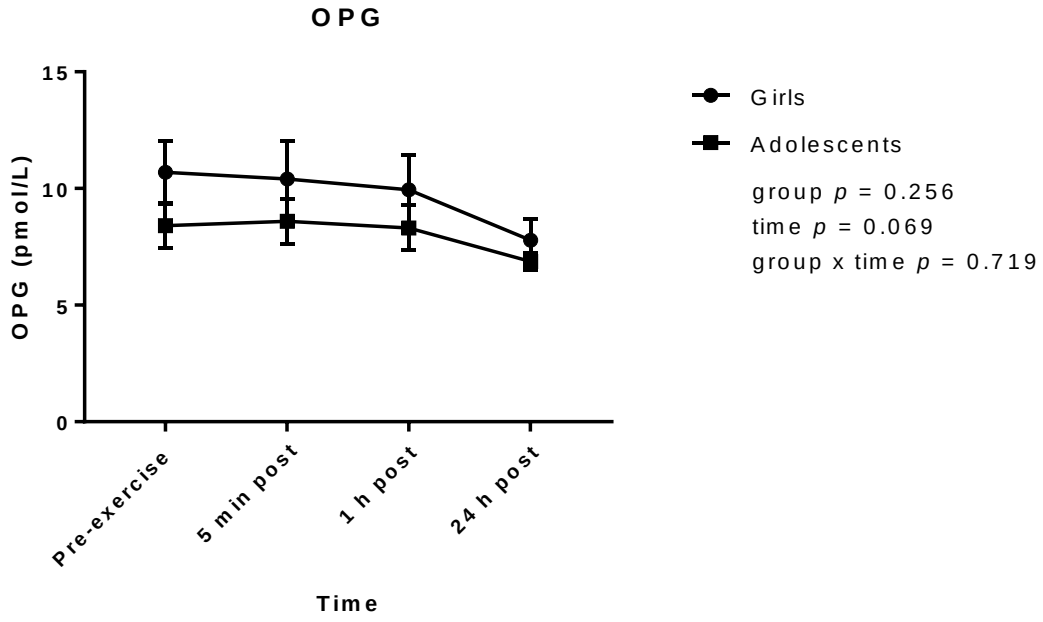

Figure 7. OPG response to the plyometric exercise in girls and adolescents.
All values are presented as mean ± standard error.

There was no significant interaction (p>0.05) and no group effect (p>0.05) found for OPG. However, there was a trend towards a significant effect for time (p=0.069) (Figure 7). The observed power for OPG was 0.480.

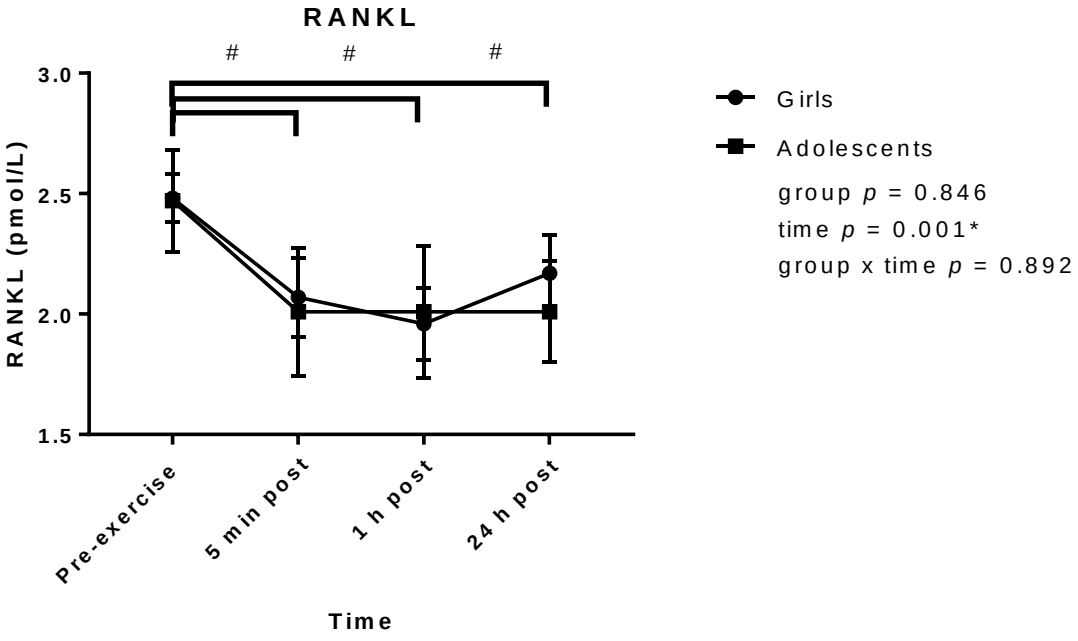

Figure 8. RANKL response to the plyometric exercise in girls and adolescents.
All values are presented as mean ± standard error.
# indicates a significant difference between time points

There was no significant interaction ($p > 0.05$) and no a significant group effect for RANKL ($p>0.05$). The effect for time was significant ($p=0.001$) with post hoc analysis revealing a significant overall decrease 5 min post exercise ($p=0.014$) that remains lower 1 hour ($p=0.048$) and 24 hours ($p=0.012$) following exercise compared to pre-exercise values in both groups (Figure 8). The observed power for RANKL was 0.997.

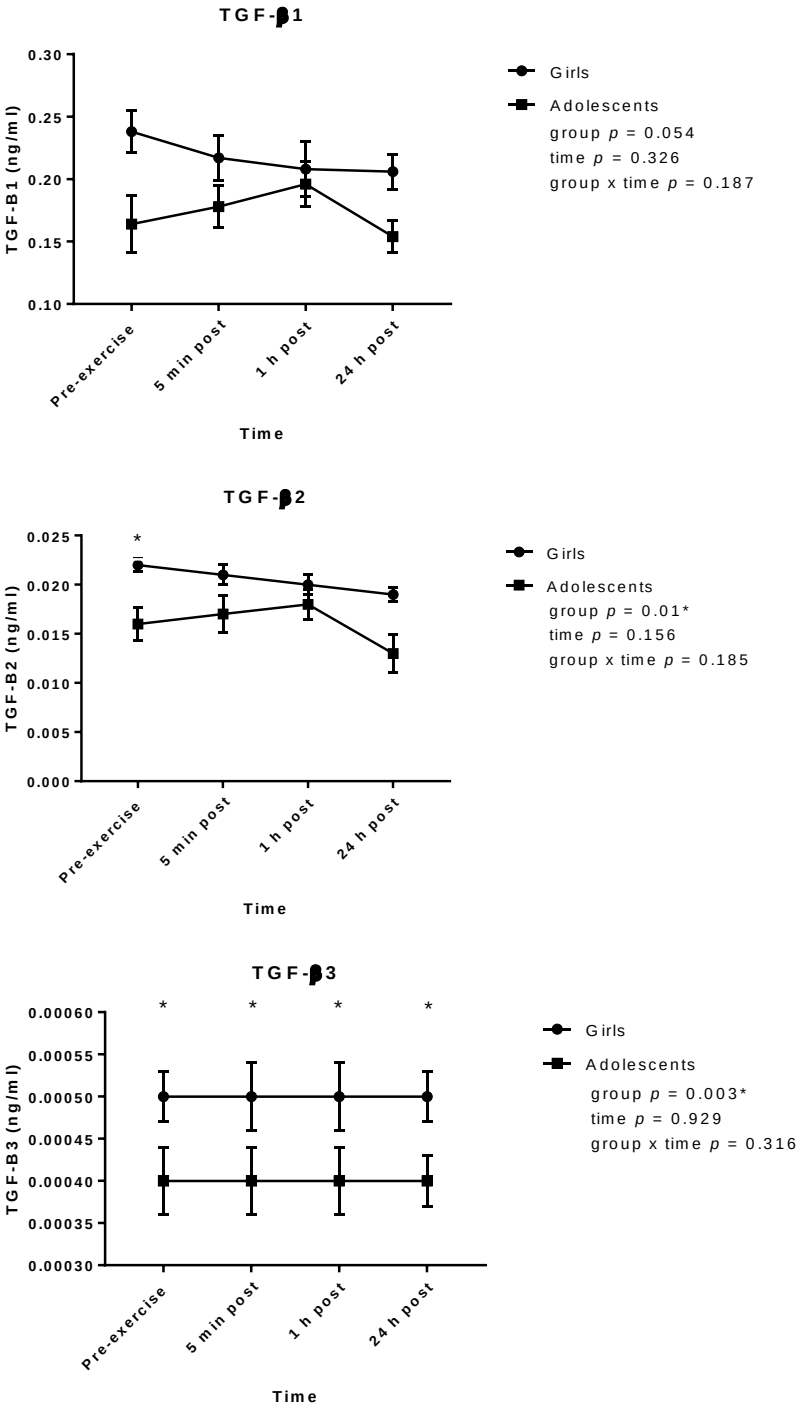

Figure 9. TGF- β1 (a), TGF- β2 (b) and TGF- β3 (c) response to plyometric exercise in girls and adolescents.

All values are presented as mean ± standard error

*indicates a significant difference between groups

There was no interaction (p>0.05), group (p>0.05) or time effect (p > 0.05) for TGF- β1 (Figure 9a). There was no interaction (p > 0.05), or time effect (p > 0.05) for TGF-β2, however the group effect was significant (p=0.010) with post hoc analysis revealing significantly higher levels in the girls at baseline (p=0.002) (Figure 9b). There was no interaction effect (p>0.05), or time effect (p>0.05) for TGF-β3, but there was a significant main effect for group (p=0.003) with the post hoc analysis revealing the girls displaying higher levels at baseline (p<0.0001), 5 min (p<0.0001), 1 hour and 24 hours (p<0.0001) post exercise (Figure 9c). The observed power for TGF-β1, TGF-β2 and TGF-β3 was 0.619, 0.176 and 0.549 respectively.

Chapter 5: Discussion

This is the first study to examine the response of bone biomarkers (i.e., osteokines and transforming growth factors) associated with the anabolic Wnt signalling and the catabolic RANKL pathway in girls and adolescent females to a plyometric exercise session. The main findings of this study are as follows: (1) girls had higher resting levels of DKK-1, TGF-β1, TGF-β2 and TGF-β3; (2) DKK-1 significantly decreased 24 hours following exercise and RANKL decreased 5 min post exercise and remained suppressed 1 hour and 24 hours following exercise while sclerostin, OPG and all three isotopes of TGF-β were unresponsive to exercise in both girls and adolescents; and (3) the direction and timing of the exercise induced changes are not significantly different between groups for OPG, RANKL and DKK-1.

5.1 Physical Activity and Nutrition

There were no significant differences between groups in METs per week as assessed by the Godin-Shephard Leisure Time Exercise Questionnaire. METs per week did not significantly correlate with baseline measures of biochemical markers in either group. Girls have significantly lower daily energy intakes as wells as lower daily intakes of calcium. The recommended dietary allowance for calcium for children aged 4-8 years is 1000 mg/day and for girls aged 9-18 years is 1300 mg/day (142). The girls fall well below the recommended dietary allowance while the adolescent group just reaches an adequate daily amount of calcium. The recommended dietary allowance for Vitamin D for females aged 1-18 years of age is 600 IUs per day (142). Though significant differences in Vitamin D intake are present, neither group comes close to the daily recommended intake. This is typical of an adult Canadian population, where it has been

shown that daily sun exposure and daily dietary intake are insufficient to meet Vitamin D requirements (143). For total energy intake the results are much the same; The girls fall below the recommended intake for active females aged 8-11 years while the adolescents meet the recommended intake (144). Since the girls are reporting approximately half the recommended daily caloric intake as well as daily calcium intake, it is possible that this group as a whole is underreporting their diet when using a food frequency questionnaire. This could also account for the fact that the Vitamin D levels in the girls are approximately half those reported by the adolescents.

5.2 Anabolic and Catabolic Osteokines and Transforming Growth Factors

In regards to the inhibitors of the Wnt signalling pathway – i.e., sclerostin and DKK-1 – our hypothesis was confirmed for DKK-1 only. DKK-1 was significantly higher in the girls compared to the adolescents whereas no group differences were seen in resting levels of sclerostin.

The sclerostin results reported here are considerably higher than those reported by Kirmani et al. (75), who investigated differences in sclerostin between males and females. However, the age range of the participants of their study was considerably wider (4-21 years) than the age rage in our study (8-16 years). Our sclerostin levels are also higher than those reported in eumenorrheic and amenorrheic athletes aged 15-21 years (76), albeit our participants are considerably younger. Our results for both groups are similar to those previously reported in healthy controls compared to HIV positive boys and girls (145), with an average age closer to our own (11 years of age). In contrast, Falk et al. (9) reported considerably lower levels of sclerostin in pre-pubertal and mid-pubertal boys, which is in contrast to the finding in the literature that males have higher levels of

sclerostin than females (18). Since such limited research exists, particularly in pre-pubertal populations, more research is needed to establish accepted baseline values for sclerostin for both males and females at different stages of development.

Two studies have reported resting DKK-1 levels in children (average ages of 11 and 13 years respectively), both of which examined DKK-1 in altered states; one study compared obese and non-obese children and the second study compared HIV positive and HIV negative children (145,146). Both studies reported combined results for boys and girls as well as for pre- and post-pubertal. Our baseline results for the girls are in line with DKK-1 results of the HIV-negative children reported by Mora et al. (145), though the adolescent group demonstrates higher levels than those reported. The opposite is true of the study done by Radetti and colleagues, where their normal weight controls had similar results to our adolescent group while the girls displayed values lower than those reported (146). Since very limited research exists on resting DKK-1 levels in children, acceptable ranges for baseline values are unavailable.

In regards to the markers involved in the RANKL pathway, our hypothesis of group differences was not confirmed. There were no differences between groups in baseline measures of OPG and RANKL. This confirms the results of Wasilewska et al., who found no group differences between girls and boys as wells as older and younger children (147). Since there is limited research available in the pediatric population, acceptable baseline ranges are currently unknown. Our OPG levels are considerably higher than those previously reported in pre-pubertal boys (10) and adolescent females (77). Our RANKL levels are considerably lower than those reported in pre-pubertal boys

(10), and slightly higher than the adolescent females reported by Lucas and colleagues (77).

Our hypothesis of group differences was confirmed for all three isotopes of TGF-β. Specifically, TGF-β1, TGF-β2 and TGF-β3 were all significantly higher in the girls than the adolescents. We hypothesized that girls would display higher levels of TGF-β simply because they have not yet reached somatic maturity (as demonstrated in age from peak height velocity). Our study is the first to compare resting levels of transforming growth factors in a healthy pediatric population. More research is needed to confirm these results as the majority of research into TGF-β is in disease states.

5.3 Exercise Response

The unresponsiveness of sclerostin to plyometric exercise confirms the results of Falk and colleagues in pre-pubertal boys (11). Furthermore, we found an overall decrease in DKK-1 over 24 hours following the plyometric exercise session. This is the first study to investigate levels of DKK-1 in response to exercise in a pediatric population and is also the first study to demonstrate the potential of plyometric exercise to suppress this Wnt inhibitor. Such suppression could potentially indicate the ability of plyometric exercise to suppress biochemical markers that inhibit Wnt signalling and therefore promote bone formation following exercise.

No changes over time were seen in the anabolic OPG. However, there was an overall decrease in the catabolic RANKL 5 min after exercise, which remained suppressed 1 hour and 24 hours following exercise. Kish and colleagues, also reported no changes in OPG after a plyometric protocol in pre-pubertal and mid-pubertal boys but in

contrast to our results, they found no changes in RANKL in pre-pubertal boys after exercise (10). These conflicting results could indicate the potential for a sex difference in the RANKL response to plyometric exercise in pre-pubertal boys when compared to girls as well as adolescent females. As RANKL is an indicator of osteoclast activity, this research potentially demonstrates the ability of plyometric exercise to suppress osteoclast activity in young females.

No changes were seen over time following exercise in all three isotopes of TGF-β. It has been reported that TGF-β1 responded 2 hours after a bout of strenuous cycling in young adult men (113). It is possible that the acute exercise intensity required for a TGF-β response in children may be greater than what was achieved in this protocol. Further research is needed to determine if indeed TGF-β is capable of responding to exercise in pediatric populations.

A single bout of plyometric exercise is, therefore, a sufficient stimulus to affect factors associated with the Wnt pathway. The decrease in DKK-1 indicates that plyometric exercise is capable of withholding inhibitors of the Wnt pathway, allowing gene transcription in favour of bone formation to occur uninhibited. On the other hand, the interesting finding that sclerostin in unresponsive to plyometric exercise in children, which was also seen in boys by Falk and colleagues (11), suggests that inhibitors of the Wnt signalling pathway are not universally suppressed by the same type of exercise. The suppression of bone resorption indicators is also demonstrated in downstream factors such as RANKL (a known indicator of bone resorption), which was decreased almost immediately after exercise and remaining suppressed for a longer period of time. This decrease in RANKL led to an increase in OPG/RANKL ratio, suggesting a shift in favour

of bone formation without a parallel, significant increase in OPG. This immediate reduction in osteoclastogenesis independent of Wnt after plyometric exercise indicates greater bone turnover being initiated by an increase in bone resorption, which may be a pre-requisite to stimulate the Wnt signalling pathway, and a subsequent shift towards bone formation, through the suppression of catabolic osteokines such as DDK-1.

Chapter 6: Conclusion

6.1 General Conclusions

This study found that in both girls and adolescent females, a single bout of plyometric exercise was sufficient to initially suppress (i.e., 5 min post-exercise) RANKL, a marker of osteoclastogenesis, which remained lower than baseline 24 hours following the exercise, when DKK-1, an inhibitor of the anabolic Wnt signalling, also dropped regardless of pubertal status. These results suggest that a single bout of plyometric exercise could be a sufficient stimulus to shift the bone anabolic/catabolic balance in girls and adolescent females. We also demonstrated group differences in DKK-1 and TGF-β1, 2 and 3 with consistently higher levels in girls than adolescents, confirming our hypothesis of younger girls having an overall of a higher bone turnover rate.

6.2 Strengths and Limitations

There are several strengths of this study. First, the participants came into the study fasted and received a standardized breakfast after the baseline blood sample. This could potentially eliminate the immediate effects of nutrition on bone markers. Though this is not the first study to investigate the response of children to plyometric exercise, the previous studies only investigated the differences between pre-pubertal boys and men (10,11). By using a female population, we can add to the current literature regarding the relationship of plyometric exercise to bone in two distinct pediatric female populations. Another strength that was not included in the Kish et al. study was the comparison of girls before and immediately after sexual maturity. Since peak bone mass is accrued in early adulthood, the inclusion of a post-pubertal group who has presumably attained adult

height but not peak bone mass give a glimpse of the potential of plyometric exercise to positively influence bone metabolism before peak bone mass is achieved. The beginning of puberty is associated with periods of large bone growth (117) and the differences in response to plyometric exercise based on sexual maturation, to our knowledge, has not been shown in previous research. We also used a protocol that has been proven to elicit a bone response (8,10,11) and can be confident that the response in osteokines and growth factors we have seen is due to this protocol and not other lifestyle factors. We also controlled for circadian rhythm variations in bone bio markers by ensuring that our participants gave their first blood draw between 8 and 9 am.

A limitation of this study is the coefficients of variation for inter- and intra-plate reliability. Though acceptable ranges for %CV for Milliplex kits are higher than traditional ELISA kits (approximately 10% for intra-plate reliability and approximately 15% for inter-plate reliability) some of our coefficients are still slightly outside this range.

6.3 Implications and Future Directions

This study has demonstrated that plyometric exercise is capable of suppressing the catabolic osteokines DKK-1 and RANKL in pediatric females regardless of pubertal status. These results could aid the specification of physical activity guidelines for pediatric females in order to attain optimal bone mineral accrual throughout changes in puberty.

We specifically aimed to address some of the gaps in the literature regarding resting values and the subsequent response to exercise of biochemical markers involved in both the anabolic Wnt and the catabolic RANKL pathways. This study has highlighted

the need for future research to confirm resting levels of serum osteokines such as sclerostin, DKK-1, OPG and RANKL in both male and female pediatric populations as well as investigate their responses to plyometric and other modes of exercise. This study was also the first to investigate resting levels of transforming growth factors in a healthy pediatric population. Further research is also needed to confirm these results as well as further investigate their role in Wnt signalling.

Although this is the first study to demonstrate the response of Wnt signalling osteokines in pediatric females, there is very little known on the response of sclerostin to an acute session of exercise in adult females. Particularly of interest is the association of sclerostin with estrogen in post-menopausal women (148). Another area of interest would be the comparison of female athletes vs non-athletes, particularly where energy deficit may play a role.

Another area of future research would be to investigate different modes of exercise on these osteokines and growth factors in both male and female pediatric populations. Cycling, for instance, does not create ground reaction forces of the same magnitude as plyometric exercise; however very high exercise intensities can be achieved through cycling which may have differing effects on the response of the growth factors and bone biomarkers examined here.

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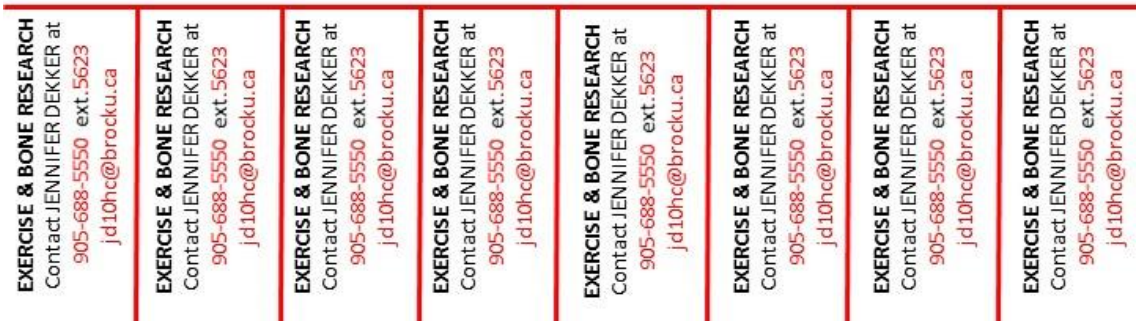
Appendix 1: Recruitment Materials

1.1 Poster



1.2 Newspaper Ad

1.3 Invitation Letter

Invitation Letter

Effects of plyometric exercise on markers of bone turnover and inflammatory cytokines in girls and women

Principal Investigator: Dr. Nota Klentrou, Faculty of Applied Health Sciences, Brock University
Co-investigators: Dr. Bareket Falk, Dr. Andrea Josse-Obar, Jennifer Dekker, Rozalia Kouvelioti, Katlynne Nelson, Kathryn Denize

If you have a female child between the ages of 8 and 16, we would like to invite you and your child(ren) to participate in this study.
The purpose of this research project is to investigate the immediate response and subsequent recovery of bone metabolism and inflammation induced by a variety of jumping exercises over a period of 24 hours in women and girls.
Tests and measurements will require 90 minutes on one day and 15 minutes on the post-exercise day. In short, measurements will include filling out several questionnaires, completing a 30 min exercise routine and measurements of bone turnover using blood samples.
Participation in this project will provide information such as height, weight, hip circumference, waist circumference and percent body fat. Participants will receive a $20 compensation for their time and travel expenses. Parking is also provided.
This research is being performed by researchers in the Applied Physiology Laboratory of Brock University.
If you are interested in participating or if you would like more information, please fill out the form on the next page and return it in the enclosed envelope. You can also email the requested information to Jennifer Dekker (Project Coordinator) at jd10hc@brocku.ca or to Nota Klentrou (Principal Investigator) at nklentrou@brocku.ca
This study has been reviewed and received ethics clearance through the Brock University Research Ethics Board (REB #14-267). If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688 5550 ext 3035 or reb@brocku.ca).

Thank you,
Dr. Nota Klentrou
Department Kinesiology
Faculty of Applied Health Science
Brock University
Tel: 905-688-5550 ext: 4538
Email: nklentrou@brocku.ca

If you are willing to be contacted to discuss having your child participate in this study, please complete one of the two following options:

OPTION 1: Fill out the information below and return this form in the enclosed envelope

OPTION 2: Send the information below to Jennifer Dekker (Project Coordinator) at jd10hc@brocku.ca

Child's first and last name:

_____

Child's age: _____

Child's sex: _____

Appendix 2: Consent Forms

2.1 Parental Consent Form

INFORMATION AND CONSENT TO PARTICIPATE IN RESEARCH

Effects of plyometric exercise on markers of bone turnover and inflammatory cytokines in girls and women

You and/or your child are being invited to participate in a research study being conducted by the investigators listed below. Prior to participating in this study please read this form to find out the purpose and tests of this study. For the tests, you will have to visit the Applied Physiology Laboratory at Brock University. This study is a part of the Faculty of Applied Health Sciences (FAHS) of Brock University.

PRINCIPAL INVESTIGATOR:	DEPARTMENT	CONTACT
Dr. Nota Klentrou	FAHS, Brock University	905-688-5550 ex. 4538

CO-INVESTIGATORS:	DEPARTMENT	CONTACT
Dr. Bareket Falk	FAHS, Brock University	905-688-5550 ex. 4979
Dr. Andrea Josse-Obar	FAHS, Brock University	905-688-5550 ex. 3502

STUDENT CO-INVESTIGATORS:	DEPARTMENT	CONTACT
Jennifer Dekker	FAHS, Brock University	905 688-5550 ex. 5623
Rozalia Kouvelioti	FAHS, Brock University	905 688-5550 ex. 5623
Katlynne Nelson	FAHS, Brock University	905 688-5550 ex. 5623
Kathryn Denize	FAHS, Brock University	905 688-5550 ex. 5623

PURPOSE

The purpose of this study is to investigate the immediate response and subsequent recovery of bone metabolism and inflammation induced by jumping exercise over a period of 24 hours in children and adults.

DESCRIPTION OF TESTING PROCEDURES

If you agree to participate in this study, you will visit our laboratory, where you and/or your child will complete one 90 minute session of testing and another session 24 hours later, lasting 15 minutes. At the end of the study you will be given a summary of the findings upon request. Shorts, a short sleeved shirt and running shoes are recommended for the measurements. Parents may be present at all stages of the study.

Participants will undergo the measurements and procedures listed below; please note that you may choose not to answer a question in any questionnaire.

Pre-exercise Assessments

1.) Participants will be asked to complete several questionnaires, outlining your general health, physical activity, nutritional habits, pubertal status and several psychological variables. The general health questionnaire screens if participants can safely participate in all of the required measurements, and offers us a better understanding of each participant's general health that helps us properly interpret the data. Please note that this questionnaire includes questions about smoking, alcohol consumption and drug use. In all questionnaires, participants may choose not to answer any question. The questionnaire used to measure pubertal status involves the participants looking at drawings of genetalia and deciding which stage of puberty they best match. This will be carried out in private to avoid any unease. The questionnaire regarding psychological variables includes questions asking about exercise and healthy eating motivations, physical self-perception and self-confidence regarding exercise and nutrition.

2.) Body Composition: we will measure height, weight, hip circumference, waist circumference and percent body fat. Percent body fat will be estimated using bioelectrical impedance analysis (BIA) and the BodyMetrix system. The BIA assessment requires the participant to stand on a weight scale and grasp handles. A mild electrical current (50kHz, 800µA) will pass through hands to feet. This current cannot be felt and causes no harm. Valid measurements require abstinence from consuming caffeine and alcohol for 6 hours prior as well as refrain from vigorous or high impact exercise for a minimum of 24 hours prior to exercise testing. The BodyMetrix system uses ultrasound to accurately measure fat thickness at the thigh, waist and tricep. These measures are then used to calculate Body Fat % and weight distribution. There is no discomfort associated with this measurement.

3.) A total of four (4) blood samples will be collected to assess biochemical markers of bone turnover and inflammatory cytokines: pre-exercise, 5 min post exercise, 60 min and 24 hours post exercise. The blood samples will be drawn by a nurse or trained member of the research team using a standard technique. Up to 20 ml of blood will be withdrawn. It should be noted that the venous blood drawing procedure is a routine procedure performed by a nurse and offers minimal risk to

participants. In rare instances, participants may experience slight pain and/or tingling in the area and/or a minor bruise from the needle. However, with the use of anaesthetic creams (e.g., Emla), which we use in the laboratory, any sensation of pain is minimal. Once samples are collected, we split them into air-tight smaller tubes and stored in a freezer until analysis. The freezer is located in a locked laboratory, which is only accessible to qualified personnel. We will hold onto these tubes for 5 years at which time they will be disposed by qualified, trained personnel and according to biosafety and University protocols.

Exercise Protocol

A heart rate monitor and accelerometer will be worn by the participant for the entire duration of the exercise session in order to assess intensity of the performed activity. The exercise session begins with a warm-up that includes 5 min of low intensity jogging and a series of dynamic stretches. The exercise protocol has been designed to provide high-impact, weight bearing loads in the form of circuit training stations. Each participant will be instructed to rotate through each of the five stations (Jumping jacks, lunge jumps, hopping, jumping over obstacles such as a bench and drop jumps) three times for a total of three sets. Jump height will be tailored to each participant based on body size. The participant will be at each station for approximately 2 minutes and the participant will then be instructed to move to the next station. A recovery period of 2 minutes will be given between each set. All exercise testing will be done in the presence of 2 study personnel, a tester and a spotter.

CONFIDENTIALITY

All data collected though this study will remain confidential and will be stored in locked offices and secured computers to which only the principal and co-investigators has access. You should be aware that the results of this study will be made available to other scientists though publication in a scientific journal, but your name and personal data will not appear in the compilation or publication of these results. A master list will be kept to link participants' names with codes. This list and all data will be kept for 5 years after the date of publication, at which time all information will be confidentially destroyed. Additionally, you will have access to your own data, as well as group data when it becomes available and if you are interested. This can easily be provided to you by contacting the principal investigators.

SECONDARY USE OF DATA

Some of this data may be of use in the future for comparative purposes by colleagues, students or other researchers. The data used by these future researchers will remain anonymized, as all personal identifiers will have been removed. You may refuse to

allow your information to be used in the future and still remain a subject in this study. In this case, your data will be confidentially destroyed 5 years after the date of publication.

Do you want your or your child's data to be used anonymized (i.e., de-identified) **in a future study?**

Please check one box: □ Yes, I want my data to be kept anonymized for future studies.

□ No, I do not want my data to be kept for future studies.

PARTICIPATION AND WITHDRAWL

Participants can choose whether or not to participate in this study and may remove their data from the study if they wish. They may do so at any time by contacting the principal investigator in writing (email or mail). Participants will receive pro-rated compensation in the event they withdraw. Participants may also refuse to answer any questions posed to them during the study and will still remain a subject in the study.

RISKS AND BENEFITS

Participation in this study will allow participants to become exposed to a research protocol, contribute to the advancement of science and gain personal and general knowledge about their own body. All the results will be provided to you upon request.

The only foreseeable risks involved in participation include:

a) Possible muscle soreness, muscle fatigue and/or joint pain within 48 hours of the exercise tests. This is likely to occur if your child is not a usual exerciser. If this does occur, it is only temporary and will dissipate within 2-3 days. Plyometric exercise involves high impact forces so a low risk for minor injury from the jumping exercise also exists. To minimize the risk, a spotter will be present at all time. Also, for any reason, the participant can request to not do a particular exercise if she does not want to. Our goal is to complete all the exercises, but it is also to maintain a positive and encouraging environment during testing. We will always ask how your child is doing following each exercise.

b) In rare instances, participants may experience slight pain and/or tingling in the area and/or a minor bruise from the venous blood draw. Children and youth are also at a risk of fainting at the sight of or introduction of a needle. However, for the younger participants a butterfly needle will be used that does not look like a typical needle. Both the nurse and researcher will show the younger participants the butterfly needle and explain the procedure. Participants who are not allergic to medications may choose to use an aesthetic cream that usually has no side effects.

However, there is a small risk for minor effects such as burning, swelling, itching, or skin rash at application site.

RIGHTS OF RESEARCH PARTICIPANTS

You will receive a signed copy of this consent form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. This study has been reviewed and received clearance from the Brock University Research Ethics Board (file # REB 14-267). If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca).

INFORMATION

Please contact Dr. Nota Klentrou at 906 688 5550 ext 4538, nklentrou@brocku.ca or Jennifer Dekker at 905 688 5550 ext 5623, jd10hc@brocku.ca if you have any questions about this study.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE ALSO RECEIVED A SIGNED COPY OF THE INFORMATION AND CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY.

_____ _____

SIGNATURE OF PARENT/GUARDIAN DATE

_____

PRINTED NAME OF PARTICIPANT

_____ _____

WITNESS DATE

In my judgment the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

_____ _____

SIGNATURE OF INVESTIGATOR DATE

2.2 Child Assent Form

Child Assent to Participate in an Exercise and Bone Research Study

Principal Investigator: Dr. Nota Klentrou, Faculty of Applied Health Sciences

Co-investigators: Dr. Bareket Falk, Dr. Andrea Josse-Obar, Jennifer Dekker, Rozalia Kouvelioti,

Katlynne Nelson, Kathryn Denize

This form may contain words you do not understand. Please ask the study staff to explain anything you do not understand.

What is this study about?

We are doing a research study, which is a special way to find out how your body works. Your mom, dad or guardian knows I am going to ask you to join this study. We are trying to find out if a series of jumping exercises will result in stronger bones.

On the first day we will ask you to come to Brock University where you will perform jumping exercises and you will complete various measurements for 90 minutes. No one outside our group will know the results of these measurements or the answers from your questionnaires. The next day you will come to the University for 15 minutes to give a blood sample.

Do I have to be in this study?

You do not have to be in this study if you don't want to.

If you don't want to participate you can stop at any time but please let the study team know. There will be no bad feelings if you do not want to do this.

If you want to be in this study, you will be asked to sign this form.

What will happen in the study?

On the first day we will ask you to complete various measurements such as height, weight, hip circumference, waist circumference and percent body fat and filling out several questionnaires. We will then take you through a series of jumping exercises. The exercise begins with 5 minutes warm-up that including jogging and stretching. Then you

rotate through each of five stations doing different jumps (jumping jacks, drop jumps, jumping over a bench etc.). You will do this three times for a total of three sets. You can rest between sets. The exercises are safe and will be done with the help of a demonstrator and a spotter. A nurse or trained member of the research team will take one blood sample before the exercise, and two being taken after the exercise. On the second day, we will ask for one more blood sample so we are able to see if your bones have changed following the jumping exercises.

The questionnaires will ask for information about your health and physical activity, what you usually eat and how mature you are and how you feel about exercise and nutrition. The questionnaire used to assess how mature you are involves looking at drawings of private parts of your body and deciding which stage of you best match. You will do this alone in a private room and you will place the page in an envelope. In all questionnaires you may choose not to answer any question and you can still be a participant in this study. No one outside our group will know the results from these measurements or the answers from your questionnaires.

Are there good things and bad things about participating in the study?

All of measurements are safe but you may experience some discomfort during the blood sample but not for long. If you want, we can put a cream on your arm so that you do not feel any pain. Your muscles may be sore after the jumping exercise. The exercises are safe but sometimes you can get small injuries from jumping. This is why a spotter will be there to assist you. You can also skip any exercise that may hurt you.

Who will know that you are in the study?

We will write papers about what we will find out and share the information with other researchers, but will not use your name when we talk about the results.

Do you want to be in this study? Is this OK?

Please check one box: □ Yes, I want to be in the study.

□ No, I do not want to be in the study.

I understand that I will receive a signed copy of this form.

Name of child (please print): _____

Signature of child: _____

Date: _____

Investigators Signature: _____

Date: _____

Appendix 3: Questionnaires

3.1 Medical History Questionnaire

SUBJECT SCREENING AND MEDICAL HISTORY QUESTIONNAIRE

Name: _____ Date: _____

Date of Birth: _____

Your responses to this questionnaire are confidential. If you answer "YES" to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators. You may refuse to answer any of the following questions.

1. Have you ever had any major joint instability or ongoing chronic pain such as in the knee, back or elbow?	**YES**	**NO**
2. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?	**YES**	**NO**
3. Have you taken any medication in the past six months?	**YES**	**NO**
4. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. asthma, diabetes, anorexia)?	**YES**	**NO**
5. Do you, or have you in the past, consumed any alcohol on a regular basis?	**YES**	**NO**
6. Do you, or have you in the past, smoked on a regular basis?	**YES**	**NO**
7. Are you, or have you in the past, engaged in any extreme diet?	**YES**	**NO**
8. Have you had any fractures?	**YES**	**NO**

_____

_____

_____

_____

3.2 Godin Shephard Leisure Time Exercise Questionnaire

GODIN-SHEPHARD LEISURE-TIME EXERCISE QUESTIONNAIRE

1. Considering a **7-day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your **free-time** (write on each line the appropriate number)?

Times Per Week

(a) STRENUOUS EXERCISE
(HEART BEATS RAPIDLY)

_____
(i.e. running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

(b) MODERATE EXERCISE
(NOT EXHAUSTING)

_____
(i.e. fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)

(c) MILD EXERCISE
(MINIMAL EFFORT)

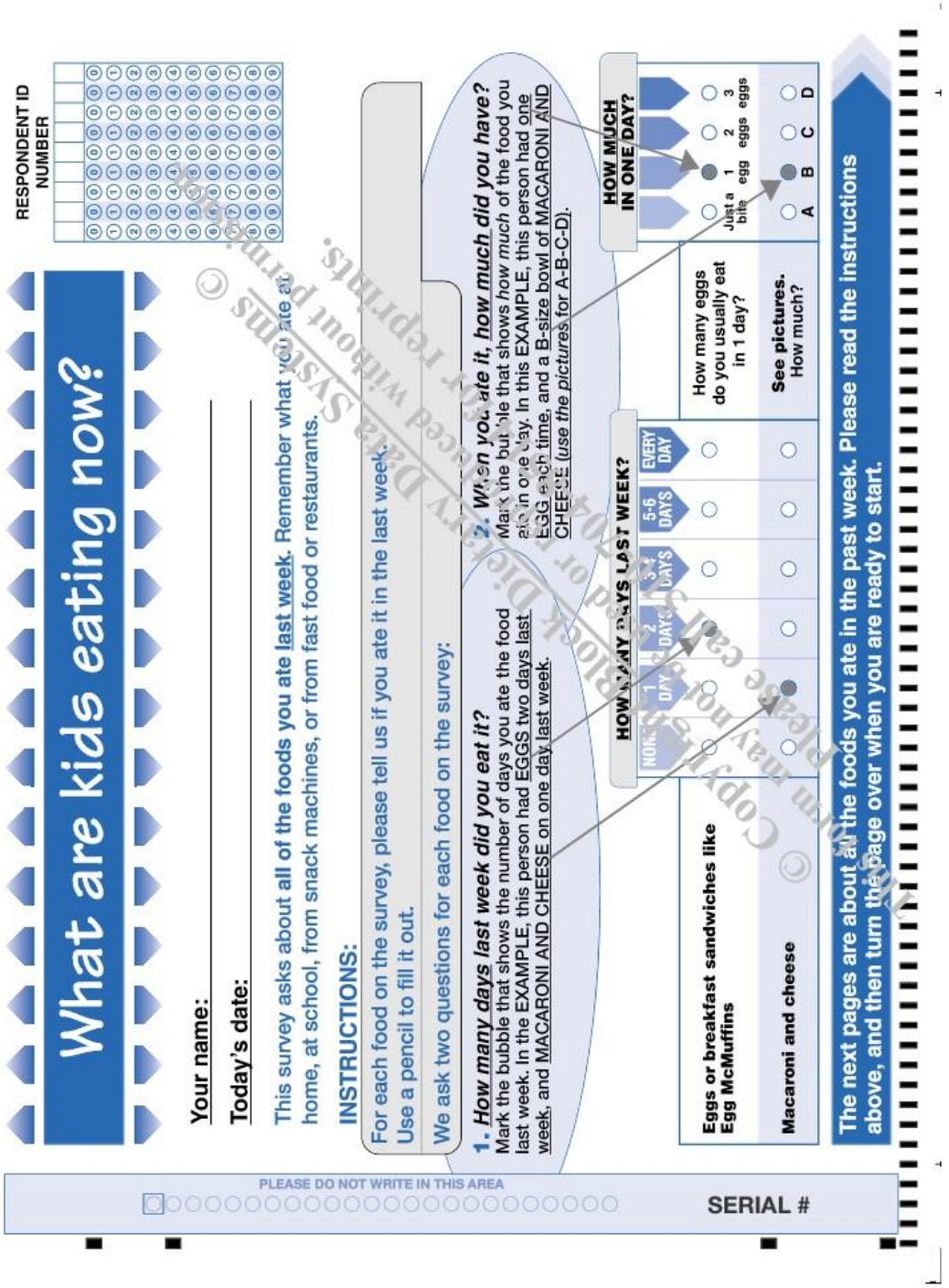
_____
(i.e. yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)

2. Considering a **7-day period** (a week), during your leisure-time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

1. OFTEN	**2. SOMETIMES**	**3. NEVER/RARELY**
☐	☐	☐

3.3 Food Frequency Questionnaire

3.3.1 BLOCK Kids



Think about every time you ate anything in the past week. You can tell us you didn't eat a food at all in the past week, or that you ate it one day last week, two days last week, 3-4 days, 5-6 days, or every day.

Remember what you ate at **home**, at **school**, from **fast food**, or from a **restaurant**.

	HOW MANY DAYS LAST WEEK? NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY		HOW MUCH IN ONE DAY?
Pancakes, waffles, Pop Tarts	○	○	○	○	○	○	How many?	○ ½ ○ 1 ○ 2 ○ 3
Granola bars, breakfast bars	○	○	○	○	○	○	How many?	○ ½ ○ 1 ○ 2 ○ 3
Eggs or breakfast sandwiches like Egg McMuffins	○	○	○	○	○	○	How many eggs do you usually eat in 1 day?	○ Just a bite ○ 1 egg ○ 2 eggs ○ 3 eggs
Bacon or sausage	○	○	○	○	○	○		
Cooked cereal like oatmeal or grits	○	○	○	○	○	○	**See pictures.** Which bowl?	○ B ○ C ○ D
Cold cereal, like Corn Flakes, Frosted Flakes or any other kind	○	○	○	○	○	○	**See pictures.** Which bowl?	○ B ○ C ○ D
When you ate cereal, which kind did you eat? (MARK THE ONE YOU ATE THE MOST OF)	○ Sweet cereals like Frosted Flakes, Froot Loops ○ Plain cereals like Corn Flakes, Cheerios, Rice Krispies ○ Fiber cereals like Raisin Bran, Shredded Wheat ○ Fortified cereals like Total or Product 19							
How often do you have milk on cereal?	○	○	○	○	○	○		
Bananas	○	○	○	○	○	○	How many do you usually have in 1 day?	○ ½ ○ 1 ○ 2 ○ 3
Apples or pears	○	○	○	○	○	○	How many do you usually have in 1 day?	○ ½ ○ 1 ○ 2 ○ 3
Oranges or Tangerines (Don't count juice)	○	○	○	○	○	○	How many, in one day?	○ ½ ○ 1 ○ 2 ○ 3
Strawberries or other berries	○	○	○	○	○	○	**See pictures.** How much?	○ A ○ B ○ C ○ D

Remember what you ate at **home**, at **school**, from **fast food**, or from a **restaurant**.

	HOW MANY DAYS LAST WEEK?							HOW MUCH IN ONE DAY?			
	NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY					
Applesauce, fruit cocktail or pineapple slices	○	○	○	○	○	○	**See pictures.** Which bowl?		○ B	○ C	○ D
Any other fruit, like grapes, peaches, watermelon, cantaloupe, fruit roll-ups	○	○	○	○	○	○	**See pictures.** How much do you usually eat?	○ A	○ B	○ C	○ D
Hamburgers or cheeseburgers, at home or from a fast food restaurant	○	○	○	○	○	○	How much?	○ ½ small burger	○ 1 small burger	○ 1 large burger	○ 2 large burgers
Which kind do you usually eat?	○ hamburgers			○ cheeseburgers							
Tacos, burritos or enchiladas	○	○	○	○	○	○	How many?	○ ½	○ 1	○ 2	○ 3
Which kind of tacos, burritos, enchiladas do you usually eat?	○ with meat or chicken			○ without meat or chicken							
Hot Pockets, meat ball subs or Sloppy Joes	○	○	○	○	○	○					
Roast beef, or steak	○	○	○	○	○	○	How much?	○ A	○ B	○ C	○ D
Hamburger Helper, beef and noodles, beef stew, or any other beef dishes	○	○	○	○	○	○	How much?	○ A	○ B	○ C	○ D
Pork chops, ribs, or cooked ham	○	○	○	○	○	○	How much?	○ A	○ B	○ C	○ D
Fried chicken including chicken nuggets, from home or from a restaurant like KFC	○	○	○	○	○	○	How many pieces?	○ 1	○ 2 (or 6 nuggets)	○ 3	○ 4
Any other kind of chicken, like roasted chicken, chicken stew, Chicken Helper	○	○	○	○	○	○	How much?	○ A	○ B	○ C	○ D

SERIAL #

PLEASE DO NOT WRITE IN THIS AREA

Remember what you ate at **home**, at **school**, from **fast food**, or from a **restaurant**.

	HOW MANY DAYS LAST WEEK? NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY		HOW MUCH IN ONE DAY?
Any kind of fish, like fish sandwich, fish sticks, shrimp or tuna	○	○	○	○	○	○	How much?	○ A ○ B ○ C ○ D
Spaghetti, ravioli or lasagna <u>with tomato sauce</u>?	○	○	○	○	○	○	How much?	○ A ○ B ○ C ○ D
Macaroni and cheese	○	○	○	○	○	○	How much?	○ A ○ B ○ C ○ D
Pizza or pizza pockets	○	○	○	○	○	○	How many slices?	○ ½ ○ 1 ○ 2 ○ 3
Hot dogs or corn dogs	○	○	○	○	○	○	How many?	○ ½ ○ 1 ○ 2 ○ 3
Lunch meat like boloney, chicken, sliced ham. Remember sandwiches and Lunchables	○	○	○	○	○	○	How many slices of lunch meat?	○ ½ ○ 1 ○ 2 ○ 3
Refried beans	○	○	○	○	○	○	How much?	○ A ○ B ○ C ○ D
Vegetable <u>soup</u>, vegetable beef <u>soup</u>, or tomato <u>soup</u>	○	○	○	○	○	○	See pictures. Which bowl?	○ B ○ C ○ D
Any other soup like chicken noodle, Cup-a-soup, ramen noodles, or menudo, posole	○	○	○	○	○	○	See pictures. Which bowl?	○ B ○ C ○ D
Biscuits or muffins	○	○	○	○	○	○	How many?	○ ½ ○ 1 ○ 2 ○ 3
<u>Whole wheat</u> bread or whole wheat rolls	○	○	○	○	○	○	How many slices in one day?	○ 1 ○ 2 ○ 3-4 ○ 5 or more
<u>White bread</u>, toast or rolls, including sandwiches or bagels	○	○	○	○	○	○	How many slices in one day?	○ 1 ○ 2 ○ 3-4 ○ 5 or more

Remember what you ate at **home**, at **school**, from **fast food**, or from a **restaurant**.

	HOW MANY DAYS LAST WEEK?							HOW MUCH IN ONE DAY?			
	NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY					
Tortillas	○	○	○	○	○	○	How many ?	○ 1	○ 2	○ 3-4	○ 5 or more
Margarine or butter, like on bread or on pancakes or on potatoes	○	○	○	○	○	○	How many times each day ?	○ 1	○ 2	○ 3	○ 4
Cheese. Remember cheese in sandwiches	○	○	○	○	○	○	How many slices of cheese each day?	○ ½	○ 1	○ 2	○ 3
Mayonnaise or sandwich spread	○	○	○	○	○	○					
Peanut butter sandwich	○	○	○	○	○	○	How many on those days?	○ ½	○ 1	○ 2	○ 3
Jelly or jam	○	○	○	○	○	○					
Sunflower seeds, peanuts or other nuts	○	○	○	○	○	○	How much in one day?	○ A	○ B	○ C	○ D
Salad with lettuce, green salad	○	○	○	○	○	○	**See pictures.** Which bowl?		○ B	○ C	○ D
Salad dressing	○	○	○	○	○	○					
Green beans, string beans or peas	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Pinto beans, black beans, chili with beans, or bean burritos	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D

SERIAL #

PLEASE DO NOT WRITE IN THIS AREA

Remember what you ate at **home**, at **school**, from **fast food**, or from a **restaurant**.

	HOW MANY DAYS LAST WEEK?							HOW MUCH IN ONE DAY?			
	NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY					
Corn or corn on the cob	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Tomatoes including on salad	○	○	○	○	○	○	How much?	○ A little	○ ½ tomato	○ 1	○ 2
Greens like collards, mustard greens or spinach	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Broccoli	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Carrots, carrot sticks or cooked carrots	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Sweet potatoes, or sweet potato pie	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
French fries, Tater Tots, hash browns or home fries	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B (McD Small)	○ C (McD Large)	○ D (McD Super)
Any other kind of potatoes, like mashed, baked or boiled	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
<u>Any other</u> vegetables, like squash, cauliflower or green or red peppers	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Rice, including fried rice, Spanish rice, rice with beans	○	○	○	○	○	○	**See pictures.** How much?	○ A	○ B	○ C	○ D
Ketchup, salsa, or barbecue sauce	○	○	○	○	○	○					

Remember what you ate at **home**, at **school**, from **snack machines**, at the **movies**, or from **fast food**.

	HOW MANY DAYS LAST WEEK?							HOW MUCH IN ONE DAY?			
	NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY					
Snack chips like potato chips, tortilla chips, Doritos, popcorn, Bugles	○	○	○	○	○	○	How much in the whole day?	○ A	○ B	○ C	○ D
Crackers, including snack crackers like Cheez-its, Ritz Bits, Goldfish	○	○	○	○	○	○	How much in the whole day?	○ A	○ B	○ C	○ D
Nachos with cheese	○	○	○	○	○	○	How much?	○ A	○ B	○ C	○ D
Ice cream, ice cream bars or frozen yogurt	○	○	○	○	○	○	See pictures. Which bowl?		○ B	○ C	○ D
Cookies	○	○	○	○	○	○	How many cookies?	○ 1	○ 2-3	○ 4-5	○ 6+
Donuts	○	○	○	○	○	○	How many donuts?	○ ½	○ 1	○ 2	○ 3
Cake, cupcakes, Tasty Cake, Ho-Ho's, Twinkies	○	○	○	○	○	○	How many pieces?	○ ½	○ 1	○ 2	○ 3
Pie, fruit pie, fruit crisp, cobbler	○	○	○	○	○	○	How many pieces?	○ ½	○ 1	○ 2	○ 3
Chocolate candy, like candy bars, M&Ms, Reese's, Tootsie Roll	○	○	○	○	○	○	How many bars?	○ 1 small	○ 1 medium	○ 1 large	○ 2 large
Any other candy (not chocolate), like Skittles, Starburst, Lifesavers, gum	○	○	○	○	○	○	How many packages?	○ ¼	○ ½	○ 1	○ 2
Chocolate milk, hot chocolate or cocoa	○	○	○	○	○	○	How many glasses or cartons each day?	○ ½	○ 1	○ 2	○ 3
Milk (not chocolate). (Don't count milk on cereal)	○	○	○	○	○	○	How many glasses or cartons each day?	○ ½	○ 1	○ 2	○ 3
What kind of milk do you usually drink?	○ Whole milk ○ Non-fat milk ○ Soy milk		○ Reduced-fat (2%) milk ○ Lactaid milk ○ Rice milk			○ Low-fat (1%) milk ○ Don't know					

SERIAL # PLEASE DO NOT WRITE IN THIS AREA

Remember what you ate at **home**, at **school**, from **soda machines**, at the **movies**, or from **fast food**.

	HOW MANY DAYS LAST WEEK? NONE	1 DAY	2 DAYS	3-4 DAYS	5-6 DAYS	EVERY DAY		HOW MUCH IN ONE DAY?
Sodas like Coke, Dr. Pepper, 7-Up, Sprite, Sunkist, Orange Crush. (Don't count diet sodas)	○	○	○	○	○	○	How many bottles or cans in 1 day?	○ 1 ○ 2 ○ 3-4 ○ 5+
What size soda do you usually drink?	○ 12 ounce can ○ 20 ounce bottle ○ more than 20 ounces							
Slurpees, snow cones, popsicles (not ice cream)	○	○	○	○	○	○		
Hawaiian Punch, Kool-Aid, Sunny Delight, Gatorade, ice tea, Snapple	○	○	○	○	○	○	How many glasses in 1 day?	○ 1 ○ 2 ○ 3 ○ 4
Hi-C, Tang, Tampico, Mr. Juicy, Ssips punch	○	○	○	○	○	○	How many glasses or juice boxes?	○ 1 ○ 2 ○ 3 ○ 4
Real orange juice (Don't count Sunkist or other orange sodas)	○	○	○	○	○	○	How many glasses or juice boxes?	○ 1 ○ 2 ○ 3 ○ 4
Any other real fruit juices like apple juice or grape juice. (Remember juice boxes)	○	○	○	○	○	○	How many glasses or juice boxes?	○ 1 ○ 2 ○ 3 ○ 4

In the past week, did you take any **vitamin pills**, like One-a-Day or Flintstones? ○ No ○ Yes **If yes**, how many days last week? ○ 1-2 ○ 3-4 ○ 5-6 ○ 7

Are you ○ Male ○ Female

How old are you? _____

How tall are you? ___ Feet ___ Inches

How much do you weigh? ___ Pounds

Are you (Mark all that apply)

○ African American ○ Asian
○ White ○ American Indian, Alaska native
○ Hispanic/Latino ○ Other

OFFICE USE ONLY

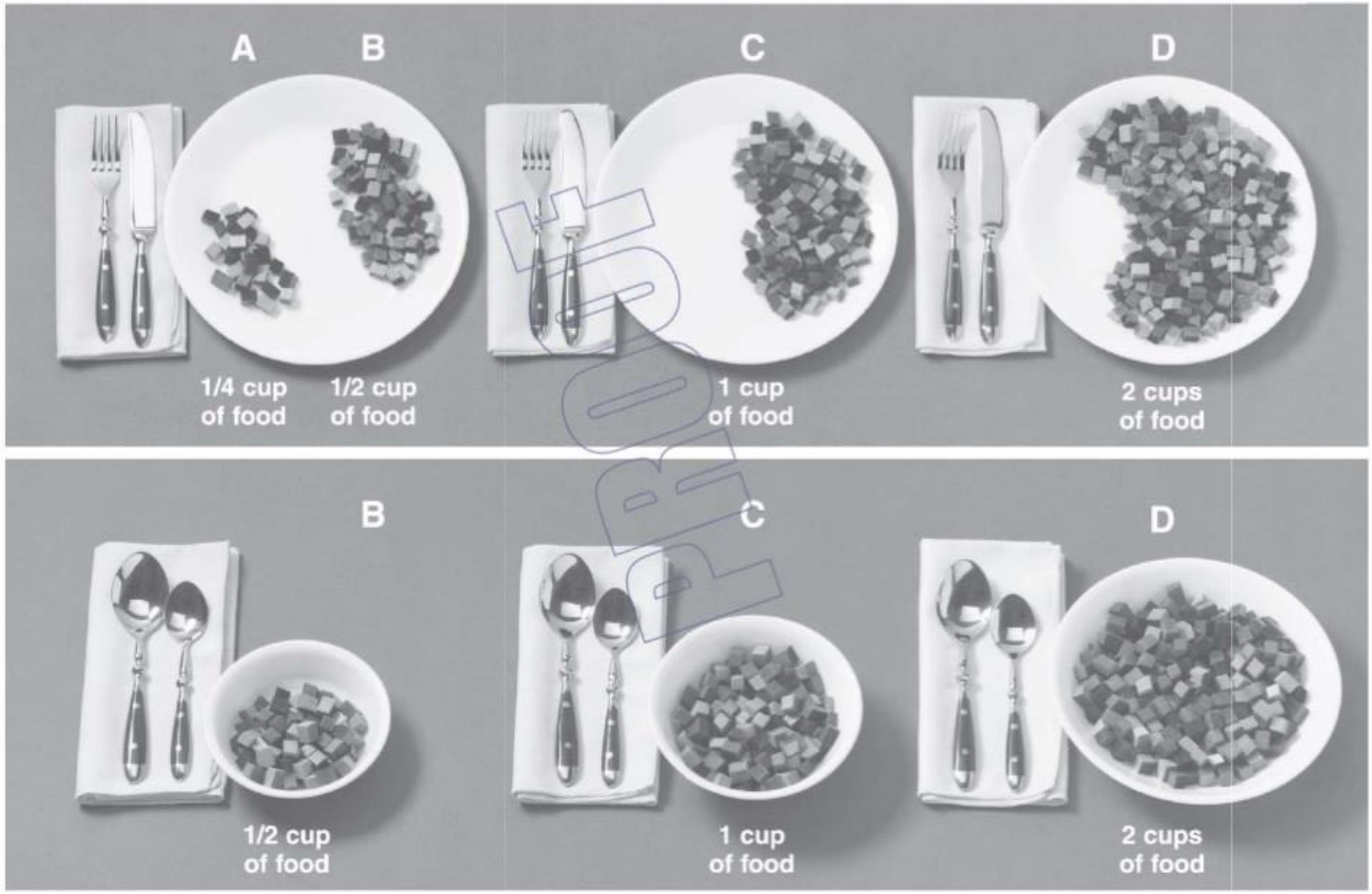
TODAY'S DATE MO.	DAY	YR.	AGE	WEIGHT pounds	HEIGHT ft. in.

1/4" SPINE PERF

Portion Size Choices

Keep this in front of you while you are filling out The Food Questionnaire. You may use either the plates or the bowls to help you choose your usual portion size.

Choose A, B, C or D: **A** = 1/4 Cup of Food **B** = 1/2 Cup of Food **C** = 1 Cup of Food **D** = 2 Cups of Food



3.3.2 Food and Activity Questionnaire 2004

FOOD AND ACTIVITY QUESTIONNAIRE

RESPONDENT ID #

TODAY'S DATE

Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

DAY

YEAR 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023

ABOUT YOU

SEX
- Male
- Female

AGE

WEIGHT pounds

HEIGHT feet inches

If female, are you pregnant or breast feeding?
- No
- Yes
- Not female

ABOUT THIS SURVEY

Please answer each question as best you can. Estimate if you aren't sure.

- **DETACH THE LAST PAGE OF THIS BOOKLET.** These are your portion pictures.
- **USE ONLY A NUMBER 2 PENCIL.**
- **FILL IN THE CIRCLES COMPLETELY** and erase completely if you make any changes.

INSTRUCTIONS

This form is about the foods you usually eat. Think about your intake over the last 6 months. This includes all meals or snacks, at home, in a restaurant, or carry-out.

Please tell us...

1. **HOW OFTEN,** on average, did you eat the food?
   DO NOT SKIP any foods. Mark "Never" if you didn't eat any of the food.
2. **HOW MUCH** of the food did you usually eat on the days you ate it?
   Sometimes we ask "how much" as A, B, C or D. **LOOK AT THE PORTION PICTURES.**
   Pick the picture that looks the most like the serving size you usually eat.
   (If you don't have pictures: A=1/4 cup, B=1/2 cup, C=1 cup, D=2 cups.)
3. **WHAT TYPE?** For some foods we ask the type (low-fat, low-sugar...) near the end of the survey.

EXAMPLE: This person drank orange juice twice a week, and had one glass each time.
Once a week this person ate a "C"-sized serving of cold cereal (about 1 cup).

	HOW OFTEN in the past 6 months?									HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D	
	NEVER	A FEW TIMES PER 6 MONTHS	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY		
Orange juice	○	○	○	○	○	●	○	○	○	How many glasses	1 ● 2 ○ 3 ○ 4 ○
Cold cereal	○	○	○	○	●	○	○	○	○	Which bowl	A ○ B ○ C ● D ○

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

Block 2014.1_6Mo ©www.NutritionQuest.com Phone 510-704-8514

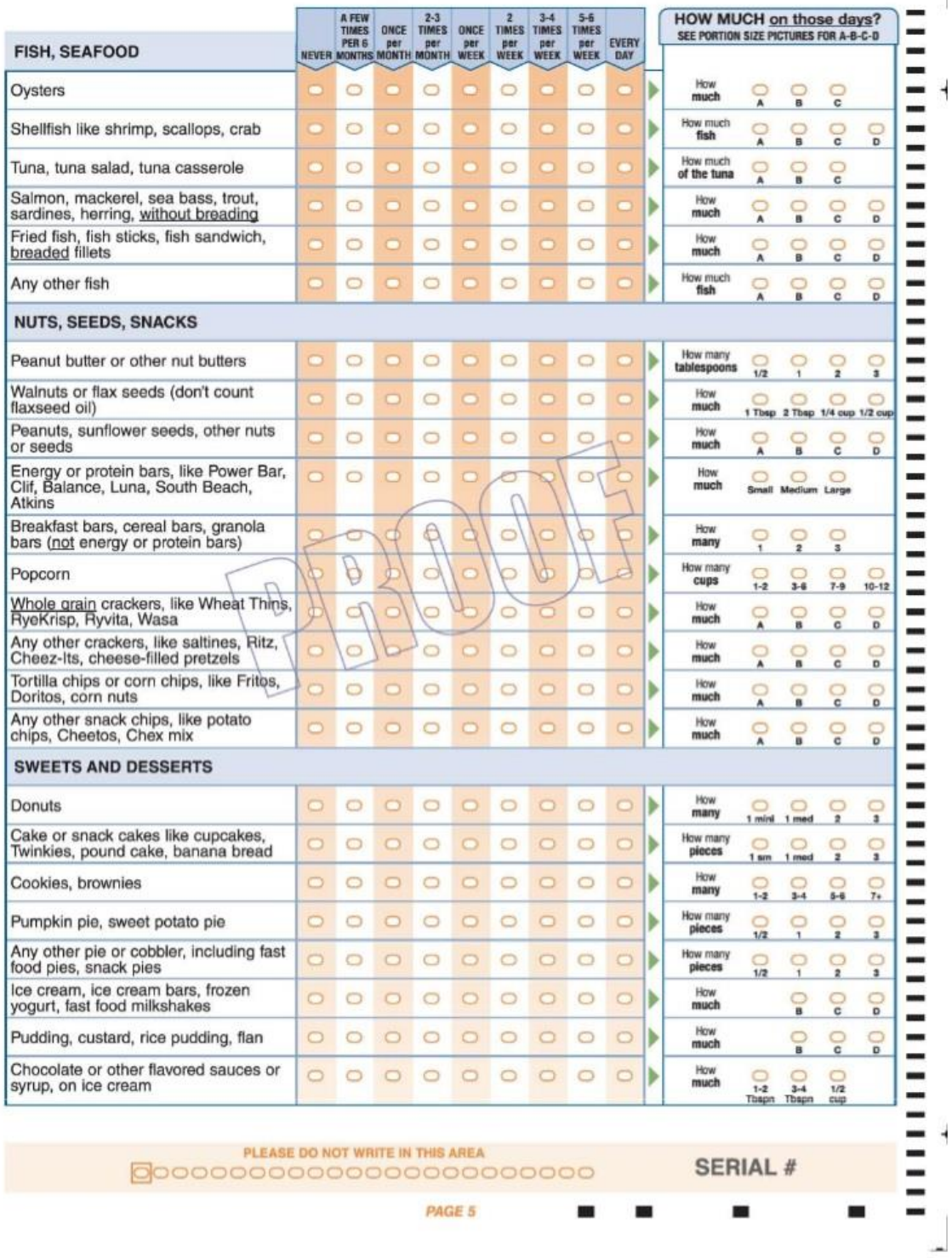
EGGS and DAIRY FOODS	NEVER	A FEW TIMES PER 6 MONTHS	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Breakfast sandwiches or breakfast burritos with eggs or meat										How many sandwiches in a day: 1, 2
Other eggs like scrambled or boiled, or quiche (not egg substitutes)										How many eggs a day: 1, 2, 3, 4
Yogurt (not frozen yogurt)										Which bowl or glass: B, C, D
Cottage cheese, ricotta cheese										How much: A, B, C, D
Cream cheese, sour cream, dips										How many tablespoons: 1, 2, 3, 4
Cheese, sliced cheese, cheese spread, including in sandwiches and quesadillas										How many slices: 1, 2, 3, 4
CEREALS, GRAINS, BREADS										
Cold cereals, ANY KIND, like corn flakes, fiber cereals, sweetened cereals										Which bowl: B, C, D
Oatmeal, or whole grain cereal like Wheatena or Ralston										Which bowl: A, B, C, D
Grits, cream of wheat, cornmeal mush										Which bowl: A, B, C, D
Milk or milk substitutes on cereal										
Brown rice, or dishes made with brown rice										How much in a day: B, C, D
White rice, or dishes made with rice, like rice and beans										How much in a day: B, C, D
Pancakes, waffles, French toast, crepes										How many: 1, 2, 3, 4
Breakfast pastries, like muffins, scones, sweet rolls, Danish, Pop Tarts, pan dulce										How many pieces: 1 sm, 1 med, 2, 3
Biscuits, not counting breakfast sandwiches										How many: 1 sm, 1 med, 2, 3
Corn bread, corn muffins, hush puppies										How many pieces in a day: 1/2, 1, 2, 3
Hamburger buns, hotdog buns, submarine or hoagie buns										How many buns in a day: 1/2, 1, 2, 3
Bagels or English muffins, dinner rolls, pita, naan										How many: 1/2, 1, 2, 3
Tortillas (not counting in tacos or burritos)										How many in a day: 1, 2, 3, 4
Any other bread or toast, including white, dark, whole wheat, and what you have in sandwiches										How many slices in a day: 1, 2, 3, 4
VEGETABLES										
Broccoli, Chinese broccoli, or Brussels sprouts										How much: A, B, C, D
Carrots and mixed vegetables containing carrots										How much: A, B, C, D
Corn										How much: A, B, C, D
Green beans, string beans, green peas										How much: A, B, C, D
Cooked greens like spinach, collards, turnip greens, kale, mustard greens										How much: A, B, C, D

	NEVER	A FEW TIMES PER 6 MONTHS	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Cabbage, cole slaw, Chinese cabbage	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Green salad with lettuce or raw spinach	○	○	○	○	○	○	○	○	○	How much ○ 1/2 cup ○ 1 cup ○ 2 cups ○ 3+ cups
Raw tomatoes	○	○	○	○	○	○	○	○	○	How much ○ 1/4 ○ 1/2 ○ 1 ○ 2
Salad dressing	○	○	○	○	○	○	○	○	○	How many tablespoons ○ 1 ○ 2 ○ 3 ○ 4
Avocado, guacamole	○	○	○	○	○	○	○	○	○	How many tablespoons ○ 1 ○ 2 ○ 3 ○ 4
Sweet potatoes, yams	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
French fries, home fries, hash browns, tater tots	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Potatoes not fried, like baked, boiled, mashed, or in stew or potato salad	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Any other vegetable, like squash, cauliflower, peppers, okra, nopales	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
FRUITS										
How often do you eat the following fruits? Include fresh or frozen fruits. Only include canned or dried fruit when mentioned.										
Watermelon, cantaloupe, honeydew, other melons	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Strawberries or other berries	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Bananas	○	○	○	○	○	○	○	○	○	How many in a day ○ 1/2 ○ 1 ○ 2
Apples or pears	○	○	○	○	○	○	○	○	○	How many in a day ○ 1/2 ○ 1 ○ 2
Oranges, tangerines, grapefruit	○	○	○	○	○	○	○	○	○	How many ○ 1/2 ○ 1 ○ 2
Peaches and nectarines	○	○	○	○	○	○	○	○	○	How many ○ 1/2 ○ 1 ○ 2
Any other fresh fruit, like grapes, plums, mango, fruit salad	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Raisins, dates, other dried fruit	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C
Canned fruit, like applesauce, fruit cocktail, canned peaches or pineapple	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
BEANS, TOFU, and MEAT SUBSTITUTES **Include those eaten alone, or in mixed dishes like burritos, chili, stir-fry, salad**										
Refried beans, bean burritos, or hummus	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Pinto beans, black beans, kidney beans, baked beans, lentils	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Tofu or tempeh	○	○	○	○	○	○	○	○	○	How much ○ A ○ B ○ C ○ D
Meat substitutes, like veggie burgers, veggie chicken, vegetarian hot dogs or vegetarian lunch meats	○	○	○	○	○	○	○	○	○	How much ○ A ○ B 1 patty or dog ○ C ○ D

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

SOUPS, MIXED DISHES, and NOODLES	NEVER	A FEW TIMES PER 6 MONTHS	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Split pea, bean, or lentil soup	○	○	○	○	○	○	○	○	○	Which bowl: B C D
Vegetable soup, vegetable beef soup, or tomato soup	○	○	○	○	○	○	○	○	○	Which bowl: B C D
Any other soup, including chicken noodle, cream soups, Cup-A-Soup, ramen	○	○	○	○	○	○	○	○	○	Which bowl: B C D
Pizza or pizza pockets	○	○	○	○	○	○	○	○	○	How many slices: 1 2 3 4
Macaroni and cheese	○	○	○	○	○	○	○	○	○	How much: B C D
Spaghetti, lasagna, other pasta with tomato sauce	○	○	○	○	○	○	○	○	○	How much: B C D
Other noodles like plain pasta, pasta salad, sopa seca	○	○	○	○	○	○	○	○	○	How much: B C D
Egg rolls, won tons, samosas, empanadas	○	○	○	○	○	○	○	○	○	How many pieces: 1 2 3 4
MEAT and CHICKEN										
Hamburgers, cheeseburgers, turkey burger, at home or from a restaurant	○	○	○	○	○	○	○	○	○	How many: 1 sm 1 lrg 2 3
Hot dogs or dinner sausage like Polish, Italian, chicken apple	○	○	○	○	○	○	○	○	○	How many: 1 2 3 4
Bacon or breakfast sausage	○	○	○	○	○	○	○	○	○	How many pieces: 1 2 3 4
Lunch meats like bologna, sliced ham, sliced turkey, salami	○	○	○	○	○	○	○	○	○	How many slices: 1 2 3 4
Meat loaf, meat balls	○	○	○	○	○	○	○	○	○	How much: B C D
Steak, roast beef, pot roast, including in frozen dinners or sandwiches	○	○	○	○	○	○	○	○	○	How much: A B C D
Tacos, burritos, enchiladas, tamales, tostadas, with meat or chicken	○	○	○	○	○	○	○	○	○	How much: A B C D
Ribs, spareribs	○	○	○	○	○	○	○	○	○	How much: A B C D
Pork chops, pork roast, cooked ham (including for breakfast)	○	○	○	○	○	○	○	○	○	How much: A B C D
Any other beef or pork dish like stew, pot pie, corned beef hash, chili, Hamburger Helper, curry	○	○	○	○	○	○	○	○	○	How much: B C D
Liver, including chicken livers or liverwurst	○	○	○	○	○	○	○	○	○	How much: A B C
Pigs feet, neck bones, oxtails, tongue, chitlins	○	○	○	○	○	○	○	○	○	How much: A B C
Veal, lamb, goat, deer meat, other game	○	○	○	○	○	○	○	○	○	How much: A B C
Fried chicken, including chicken fingers, chicken nuggets, wings, chicken patty	○	○	○	○	○	○	○	○	○	How many medium pieces: 1 2 pcs/ 6 nuggets 3 4
Roasted or broiled chicken or turkey	○	○	○	○	○	○	○	○	○	How much: A B medium piece C D half chicken
Any other chicken or turkey dish, like chicken stew or curry, chicken salad, stir-fry, Chinese chicken dishes	○	○	○	○	○	○	○	○	○	How much: B C D


FISH, SEAFOOD
NEVER
A FEW TIMES PER 6 MONTHS
ONCE per MONTH
2-3 TIMES per MONTH
ONCE per WEEK
2 TIMES per WEEK
3-4 TIMES per WEEK
5-6 TIMES per WEEK
EVERY DAY
HOW MUCH on those days?
SEE PORTION SIZE PICTURES FOR A-B-C-D
Oysters
How much A B C
Shellfish like shrimp, scallops, crab
How much fish A B C D
Tuna, tuna salad, tuna casserole
How much of the tuna A B C
Salmon, mackerel, sea bass, trout, sardines, herring, without breading
How much A B C D
Fried fish, fish sticks, fish sandwich, breaded fillets
How much A B C D
Any other fish
How much fish A B C D
NUTS, SEEDS, SNACKS
Peanut butter or other nut butters
How many tablespoons 1/2 1 2 3
Walnuts or flax seeds (don't count flaxseed oil)
How much 1 Tbsp 2 Tbsp 1/4 cup 1/2 cup
Peanuts, sunflower seeds, other nuts or seeds
How much A B C D
Energy or protein bars, like Power Bar, Clif, Balance, Luna, South Beach, Atkins
How much Small Medium Large
Breakfast bars, cereal bars, granola bars (not energy or protein bars)
How many 1 2 3
Popcorn
How many cups 1-2 3-6 7-9 10-12
Whole grain crackers, like Wheat Thins, RyeKrisp, Ryvita, Wasa
How much A B C D
Any other crackers, like saltines, Ritz, Cheez-Its, cheese-filled pretzels
How much A B C D
Tortilla chips or corn chips, like Fritos, Doritos, corn nuts
How much A B C D
Any other snack chips, like potato chips, Cheetos, Chex mix
How much A B C D
SWEETS AND DESSERTS
Donuts
How many 1 mini 1 med 2 3
Cake or snack cakes like cupcakes, Twinkies, pound cake, banana bread
How many pieces 1 sm 1 med 2 3
Cookies, brownies
How many 1-2 3-4 5-6 7+
Pumpkin pie, sweet potato pie
How many pieces 1/2 1 2 3
Any other pie or cobbler, including fast food pies, snack pies
How many pieces 1/2 1 2 3
Ice cream, ice cream bars, frozen yogurt, fast food milkshakes
How much B C D
Pudding, custard, rice pudding, flan
How much B C D
Chocolate or other flavored sauces or syrup, on ice cream
How much 1-2 Tbspn 3-4 Tbspn 1/2 cup
PLEASE DO NOT WRITE IN THIS AREA
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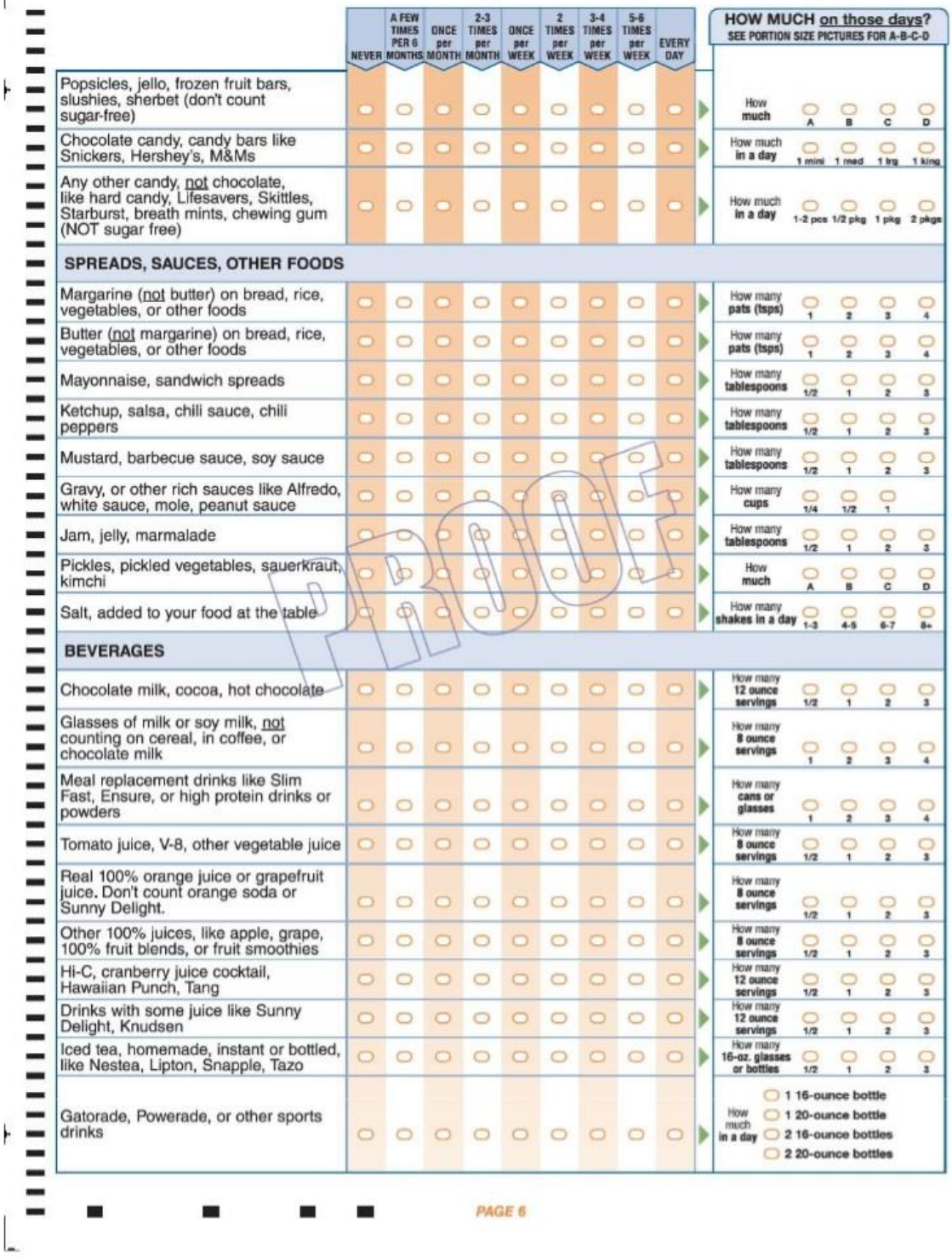
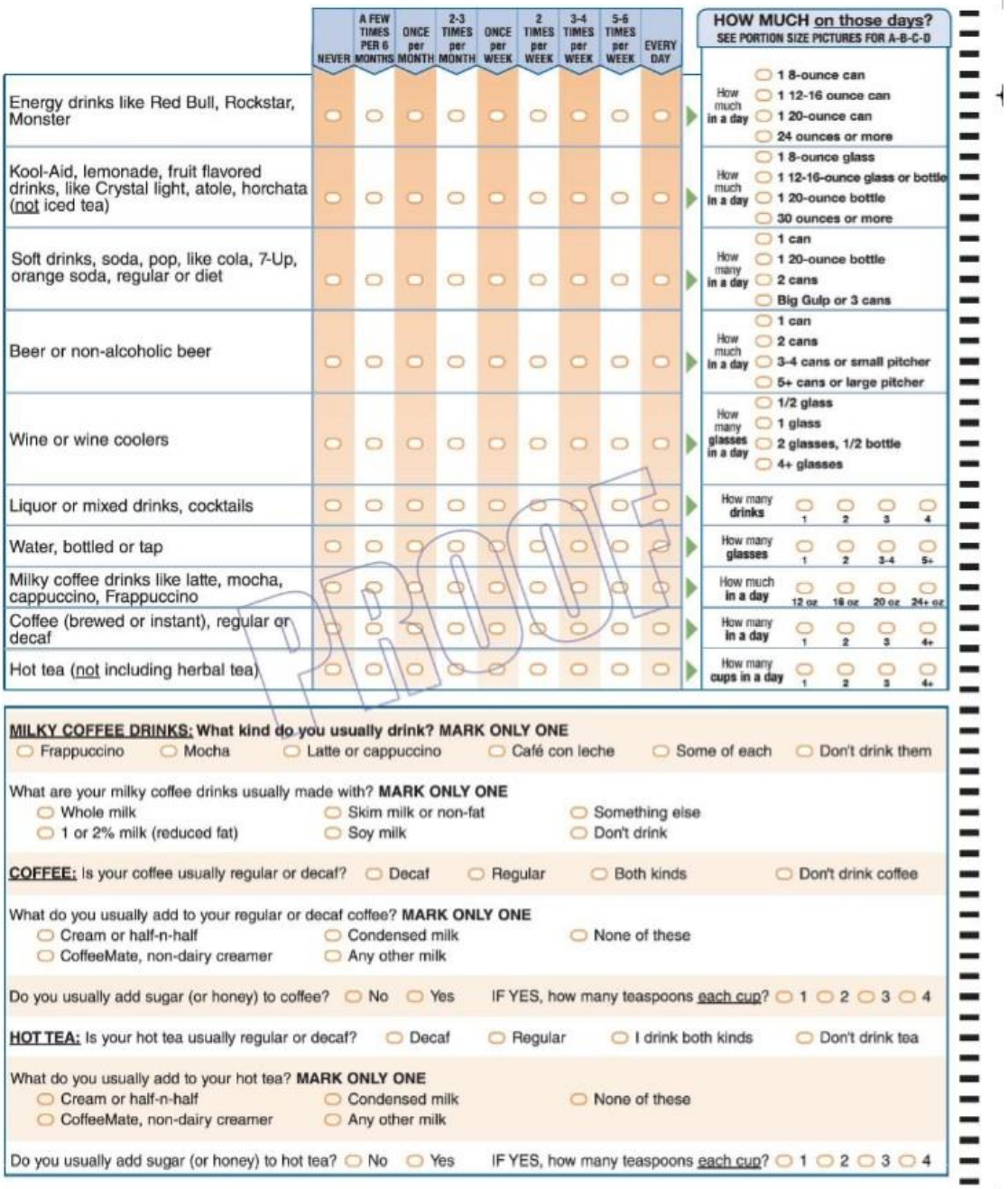
PAGE 5


NEVER
A FEW TIMES PER 6 MONTHS
ONCE per MONTH
2-3 TIMES per MONTH
ONCE per WEEK
2 TIMES per WEEK
3-4 TIMES per WEEK
5-6 TIMES per WEEK
EVERY DAY
HOW MUCH on those days?
SEE PORTION SIZE PICTURES FOR A-B-C-D
Popsicles, jello, frozen fruit bars, slushies, sherbet (don't count sugar-free)
How much A B C D
Chocolate candy, candy bars like Snickers, Hershey's, M&Ms
How much in a day 1 mini 1 med 1 lrg 1 king
Any other candy, not chocolate, like hard candy, Lifesavers, Skittles, Starburst, breath mints, chewing gum (NOT sugar free)
How much in a day 1-2 pcs 1/2 pkg 1 pkg 2 pkgs
SPREADS, SAUCES, OTHER FOODS
Margarine (not butter) on bread, rice, vegetables, or other foods
How many pats (tsps) 1 2 3 4
Butter (not margarine) on bread, rice, vegetables, or other foods
How many pats (tsps) 1 2 3 4
Mayonnaise, sandwich spreads
How many tablespoons 1/2 1 2 3
Ketchup, salsa, chili sauce, chili peppers
How many tablespoons 1/2 1 2 3
Mustard, barbecue sauce, soy sauce
How many tablespoons 1/2 1 2 3
Gravy, or other rich sauces like Alfredo, white sauce, mole, peanut sauce
How many cups 1/4 1/2 1
Jam, jelly, marmalade
How many tablespoons 1/2 1 2 3
Pickles, pickled vegetables, sauerkraut, kimchi
How much A B C D
Salt, added to your food at the table
How many shakes in a day 1-3 4-5 6-7 8+
BEVERAGES
Chocolate milk, cocoa, hot chocolate
How many 12 ounce servings 1/2 1 2 3
Glasses of milk or soy milk, not counting on cereal, in coffee, or chocolate milk
How many 8 ounce servings 1 2 3 4
Meal replacement drinks like Slim Fast, Ensure, or high protein drinks or powders
How many cans or glasses 1 2 3 4
Tomato juice, V-8, other vegetable juice
How many 8 ounce servings 1/2 1 2 3
Real 100% orange juice or grapefruit juice. Don't count orange soda or Sunny Delight.
How many 8 ounce servings 1/2 1 2 3
Other 100% juices, like apple, grape, 100% fruit blends, or fruit smoothies
How many 8 ounce servings 1/2 1 2 3
Hi-C, cranberry juice cocktail, Hawaiian Punch, Tang
How many 12 ounce servings 1/2 1 2 3
Drinks with some juice like Sunny Delight, Knudsen
How many 12 ounce servings 1/2 1 2 3
Iced tea, homemade, instant or bottled, like Nestea, Lipton, Snapple, Tazo
How many 16-oz. glasses or bottles 1/2 1 2 3
Gatorade, Powerade, or other sports drinks
How much in a day
1 16-ounce bottle
1 20-ounce bottle
2 16-ounce bottles
2 20-ounce bottles
PAGE 6


NEVER
A FEW TIMES PER 6 MONTHS
ONCE per MONTH
2-3 TIMES per MONTH
ONCE per WEEK
2 TIMES per WEEK
3-4 TIMES per WEEK
5-6 TIMES per WEEK
EVERY DAY
HOW MUCH on those days?
SEE PORTION SIZE PICTURES FOR A-B-C-D
Energy drinks like Red Bull, Rockstar, Monster
How much in a day
1 8-ounce can
1 12-16 ounce can
1 20-ounce can
24 ounces or more
Kool-Aid, lemonade, fruit flavored drinks, like Crystal light, atole, horchata (not iced tea)
How much in a day
1 8-ounce glass
1 12-16-ounce glass or bottle
1 20-ounce bottle
30 ounces or more
Soft drinks, soda, pop, like cola, 7-Up, orange soda, regular or diet
How many in a day
1 can
1 20-ounce bottle
2 cans
Big Gulp or 3 cans
Beer or non-alcoholic beer
How much in a day
1 can
2 cans
3-4 cans or small pitcher
5+ cans or large pitcher
Wine or wine coolers
How many glasses in a day
1/2 glass
1 glass
2 glasses, 1/2 bottle
4+ glasses
Liquor or mixed drinks, cocktails
How many drinks
1
2
3
4
Water, bottled or tap
How many glasses
1
2
3-4
5+
Milky coffee drinks like latte, mocha, cappuccino, Frappuccino
How much in a day
12 oz
16 oz
20 oz
24+ oz
Coffee (brewed or instant), regular or decaf
How many in a day
1
2
3
4+
Hot tea (not including herbal tea)
How many cups in a day
1
2
3
4+
PROOF
MILKY COFFEE DRINKS: What kind do you usually drink? MARK ONLY ONE
Frappuccino
Mocha
Latte or cappuccino
Café con leche
Some of each
Don't drink them
What are your milky coffee drinks usually made with? MARK ONLY ONE
Whole milk
1 or 2% milk (reduced fat)
Skim milk or non-fat
Soy milk
Something else
Don't drink
COFFEE: Is your coffee usually regular or decaf?
Decaf
Regular
Both kinds
Don't drink coffee
What do you usually add to your regular or decaf coffee? MARK ONLY ONE
Cream or half-n-half
CoffeeMate, non-dairy creamer
Condensed milk
Any other milk
None of these
Do you usually add sugar (or honey) to coffee?
No
Yes
IF YES, how many teaspoons each cup?
1
2
3
4
HOT TEA: Is your hot tea usually regular or decaf?
Decaf
Regular
I drink both kinds
Don't drink tea
What do you usually add to your hot tea? MARK ONLY ONE
Cream or half-n-half
CoffeeMate, non-dairy creamer
Condensed milk
Any other milk
None of these
Do you usually add sugar (or honey) to hot tea?
No
Yes
IF YES, how many teaspoons each cup?
1
2
3
4
PAGE 7

If you eat the following foods, what type do you usually eat? MARK ONLY ONE ANSWER FOR EACH QUESTION

Milk	○ Whole milk	○ 2% milk	○ 1% milk (low-fat)	○ Skim milk, non-fat	
	○ Soy milk	○ Rice milk	○ Almond milk, other	○ Don't drink	
Slimfast, Ensure, or high protein drinks	○ Slimfast, Ensure, regular	○ Slimfast, Ensure, light or low-carb			
	○ High protein drinks, regular	○ High protein drinks, light or low-carb	○ Don't know/Don't drink		
Real 100% orange or grapefruit juice	○ Calcium-fortified	○ Not calcium fortified	○ Don't know	○ Don't drink	
Iced tea	○ Home-made, no sugar	○ Bottled, no-sugar	○ Don't drink		
	○ Home-made, with sugar	○ Bottled, pre-sweetened			
Drinks like Kool-Aid, lemonade, Crystal Light	○ Low-calorie, sugar-free	○ Regular	○ Don't drink		
Energy drinks like Red Bull, Monster	○ Sugar-free	○ Regular	○ Don't drink		
Soft drinks, soda, pop	○ Diet, low-calorie	○ Regular	○ Don't drink		
Do they usually have caffeine?	○ Has caffeine	○ No caffeine	○ Don't drink		
Beer	○ Regular	○ Light	○ Non-alcoholic	○ Don't drink	
Wine or wine cooler	○ Red wine	○ White wine	○ Both red and white wine	○ Don't drink	
Cheese	○ Low-fat	○ Regular-fat	○ Don't eat		
Yogurt	○ Plain (no sugar or fruit)	○ With fruit or other flavors			
Yogurt	○ Low-fat	○ Non-fat	○ Regular (whole milk)	○ Don't eat	
Salad dressing	○ Low-fat, lite	○ Fat free	○ Regular	○ Oil & vinegar	○ Don't use
Spaghetti or lasagna	○ Meatless	○ With meat sauce or meatballs	○ Don't eat		
Noodles, pasta	○ Rarely whole grain	○ Sometimes whole grain	○ Usually whole grain	○ Don't know/don't eat	
Burgers	○ Hamburger	○ Cheeseburger	○ Turkey burger	○ Don't eat	
Beef or pork	○ Avoid eating the fat	○ Sometimes eat the fat	○ Often eat the fat	○ Don't eat	
Chicken or turkey	○ Avoid eating the skin	○ Sometimes eat the skin	○ Often eat the skin	○ Don't eat	
Hot dogs, dinner sausage	○ Beef or pork	○ Chicken or turkey, low-fat	○ Don't eat		
Lunch meats	○ Beef or pork	○ Chicken or turkey, low-fat	○ Don't eat		
Cakes, snack cakes, cupcakes	○ Low-sugar, low-carb	○ Low-fat	○ Regular-fat	○ Don't eat	
Cookies, brownies	○ Low-sugar, low-carb	○ Low-fat	○ Regular-fat	○ Don't eat	
Ice cream, frozen yogurt	○ Low-sugar, low-carb	○ Low-fat or frozen yogurt	○ Regular	○ Don't eat	
Energy or protein bars	○ High energy	○ High protein	○ Some of each	○ Don't know	○ Don't eat
Bagels, English muffins, rolls	○ White	○ Multi-grain	○ 100% whole wheat	○ Eat all kinds	○ Don't eat
Burger, hot dog, submarine buns	○ White	○ Multi-grain	○ 100% whole wheat	○ Eat all kinds	○ Don't eat
Bread	○ White (not whole grain)	○ 100% whole wheat	○ Don't eat		
	○ Multi-grain, rye, or other brown bread	○ Eat some of each			
Tortillas	○ Corn tortillas	○ Flour tortillas (wheat)	○ Eat all kinds or don't know	○ Don't eat	
Popcorn	○ Air popped, fat-free	○ Low-fat or Light	○ Regular	○ Caramel corn	○ Don't know ○ Don't eat
Crackers, pretzels	○ Low-fat, including RyeKrisp, rice cakes, or plain pretzels	○ Don't know			
	○ Regular-fat crackers or filled pretzels	○ Don't eat			
Mayonnaise or sandwich spreads	○ Low-fat, light	○ Regular	○ Don't eat		

If you eat **cold cereals,** what do you usually eat? Choose **ONE or TWO** that you eat most often. If you usually eat just one kind, only choose one.

○ All Bran Original	○ Cinnamon Toast Crunch	○ Grape Nuts	○ Special K, plain
○ All-Bran Complete, Complete	○ Cocoa Krispies, Pebbles, Puffs	○ Honey Bunches of Oats	○ Special K, flavors
○ Apple Jacks, Cookie Crisp	○ Corn Flakes, Corn Puffs	○ Kashi GOLEAN, Heart to Heart	○ Total
○ Bran Flakes	○ Corn Pops	○ Life	○ Wheaties
○ Cap'n Crunch	○ Fiber-One, Bran Buds	○ Lucky Charms, Fruity Pebbles	○ *Other sweet cereal*
○ Cheerios, plain or Multi-Grain	○ Froot Loops	○ Oatmeal Squares, Oat Bran	○ *Other unsweetened cereal*
○ Cheerios, Honey Nut, flavors	○ Frosted Flakes	○ Raisin Bran	○ *Other whole grain cereal*
○ Chex, Wheat	○ Frosted Mini-Wheats	○ Rice Krispies, puffed rice	○ *Other bran or fiber cereal*
○ Chex, other	○ Granola	○ Shredded Wheat	○ *Don't eat cereal*

Which **fats or oils** are used most often for **cooking or frying** (not baking) in your home? **MARK ONLY ONE OR TWO**

○ Non-stick spray or none	○ Soft tub margarine	○ Corn oil, vegetable oil and blends	○ Other oil
○ Butter or ghee	○ Low-fat margarine	○ Peanut oil	○ Don't know
○ Butter/margarine blend	○ Olive oil	○ Lard, fatback, or bacon fat	
○ Stick margarine	○ Canola oil, safflower oil	○ Vegetable shortening, Crisco	

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

What vitamin supplements do you take fairly regularly?

Multiple Vitamins. Do you take...	HOW OFTEN: DIDN'T TAKE	A FEW DAYS per MONTH	1 DAY per WEEK	2 DAYS per WEEK	3-4 DAYS per WEEK	5-6 DAYS per WEEK	EVERY DAY	FOR HOW MANY YEARS?: LESS THAN 1 YEAR	1-4 YEARS	5-9 YEARS	10+ YEARS
Prenatal vitamins	○	○	○	○	○	○	○	○	○	○	○
Regular One-A-Day, Centrum, "senior" vitamins or house brands of multiple vitamins	○	○	○	○	○	○	○	○	○	○	○
Stress-tabs or B-Complex type	○	○	○	○	○	○	○	○	○	○	○
Antioxidant combination, eye formula	○	○	○	○	○	○	○	○	○	○	○
Single Vitamins or Minerals, taken alone or in combination. Do not count what is in your multiple vitamins above.											
Vitamin A (not beta-carotene)	○	○	○	○	○	○	○	○	○	○	○
Vitamin B-6	○	○	○	○	○	○	○	○	○	○	○
Vitamin B-12	○	○	○	○	○	○	○	○	○	○	○
Vitamin C	○	○	○	○	○	○	○	○	○	○	○
Vitamin D	○	○	○	○	○	○	○	○	○	○	○
Vitamin E	○	○	○	○	○	○	○	○	○	○	○
Folic acid, folate	○	○	○	○	○	○	○	○	○	○	○
Calcium or antacids with calcium, like Tums	○	○	○	○	○	○	○	○	○	○	○
Iron	○	○	○	○	○	○	○	○	○	○	○
Zinc	○	○	○	○	○	○	○	○	○	○	○
Cod liver oil, other fish oils, omega-3, flax seed oil, algae	○	○	○	○	○	○	○	○	○	○	○
Fiber supplements like Benefiber, Metamucil	○	○	○	○	○	○	○	○	○	○	○

If you take One-A-Day, Centrum or other types of multiple vitamins, do you usually take types that

○ Contain minerals, iron, zinc, etc. ○ Do not contain minerals ○ Don't know

If you take vitamin C, how many milligrams of **vitamin C** do you usually take, on the days you take it? (Select the closest amount)

○ 100 ○ 250 ○ 500 ○ 750 ○ 1000 ○ 1500 ○ 2000 ○ 3000+ ○ Don't know

If you take vitamin E, how many IUs of **vitamin E** do you usually take, on the days you take it? (Select the closest amount)

○ 100 ○ 200 ○ 300 ○ 400 ○ 600 ○ 800 ○ 1000 ○ 2000+ ○ Don't know

If you take calcium, how many milligrams of **calcium** do you usually take, on the days you take it? (Select the closest amount)

○ 100 ○ 350 ○ 650 ○ 1250+ ○ Don't know

If you take vitamin D, how many IUs of **vitamin D** do you usually take, on the days you take it? (Select the closest amount)

○ 400 ○ 600 ○ 800 ○ 1000 ○ 2000 ○ 3000 ○ 4000 ○ 5000+ ○ Don't know

If you take omega-3 supplements, what type do you usually take? **MARK ALL THAT APPLY**

○ Fish oil ○ Flax oil, hemp oil, other seed oil ○ Krill oil ○ Algae ○ Don't know

SOME LAST QUESTIONS ABOUT YOU

	RARELY	1-2 per WEEK	3-4 per WEEK	5-6 per WEEK	1 per DAY	1 1/2 per DAY	2 per DAY	3 per DAY	4+ per DAY
About how many servings of vegetables do you eat, not counting salad or potatoes? 1 serving = 1/2 cup.	○	○	○	○	○	○	○	○	○
About how many servings of fruit do you eat, not counting juices? 1 serving = 1/2 cup or 1 medium fruit.	○	○	○	○	○	○	○	○	○
How often do you eat foods prepared at home that are cooked or fried in **fat or oil?**	○	○	○	○	○	○	○	○	○

During a regular day, how many **meals and snacks** do you usually eat?

Meals per day	○ 1	○ 2	○ 3	○ 4	○ 5+
Snacks per day	○ 1	○ 2	○ 3	○ 4	○ 5+

PHYSICAL ACTIVITY SURVEY

Think about the last 6 months. How often did you do the activities listed below?

	HOW OFTEN IN THE PAST 6 MONTHS						HOW MUCH TIME ON THOSE DAYS			
	RARELY OR NEVER	A FEW TIMES A MONTH	ONCE OR TWICE A WEEK	3-4 TIMES A WEEK	5-6 TIMES A WEEK	ALMOST EVERY DAY	LESS THAN 30 MINUTES	30-60 MINUTES	1-2 HOURS	3 OR MORE HOURS
Cooking, shopping, light cleaning like doing laundry or dusting, or running errands	○	○	○	○	○	○	○	○	○	○
Slow walking like walking the dog, or light work around the house like watering	○	○	○	○	○	○	○	○	○	○
Work on the job involving standing, like store clerk, or work involving driving (like truck driver)	○	○	○	○	○	○	○	○	○	○
Taking care of children (feeding, dressing), or moderate housework like sweeping, mopping, cleaning the tub	○	○	○	○	○	○	○	○	○	○
Weeding, raking, mowing the lawn, or light house repairs	○	○	○	○	○	○	○	○	○	○
Brisk walking, dancing, hunting or fishing, golf (NOT using a golf cart), or 'friendly' outdoor games like softball	○	○	○	○	○	○	○	○	○	○
Factory work, mechanic, restaurant work, or work involving walking, like mail carrier	○	○	○	○	○	○	○	○	○	○
Construction, painting, feeding livestock, or homecare like caring for an adult family member	○	○	○	○	○	○	○	○	○	○
Heavy work like moving boxes, heavy digging or shoveling snow, farm chores like baling hay, or other HARD labor	○	○	○	○	○	○	○	○	○	○
Exercising at the gym or at home, aerobics, weight training, jogging, or vigorous sports like basketball, soccer, tennis	○	○	○	○	○	○	○	○	○	○
Bicycling or swimming for exercise	○	○	○	○	○	○	○	○	○	○

Are you ○ Hispanic or Latino ○ Not Hispanic or Latino ○ Do not wish to provide this information

What race do you consider yourself to be? **MARK ALL THAT APPLY**

○ White ○ Asian ○ Native Hawaiian or Other Pacific Islander
○ Black or African American ○ American Indian or Alaska Native ○ Do not wish to provide this information

Thank you very much for filling out this questionnaire.
Please take a minute to go back and fill in anything you may have skipped.

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

1/4" SPINE PERF

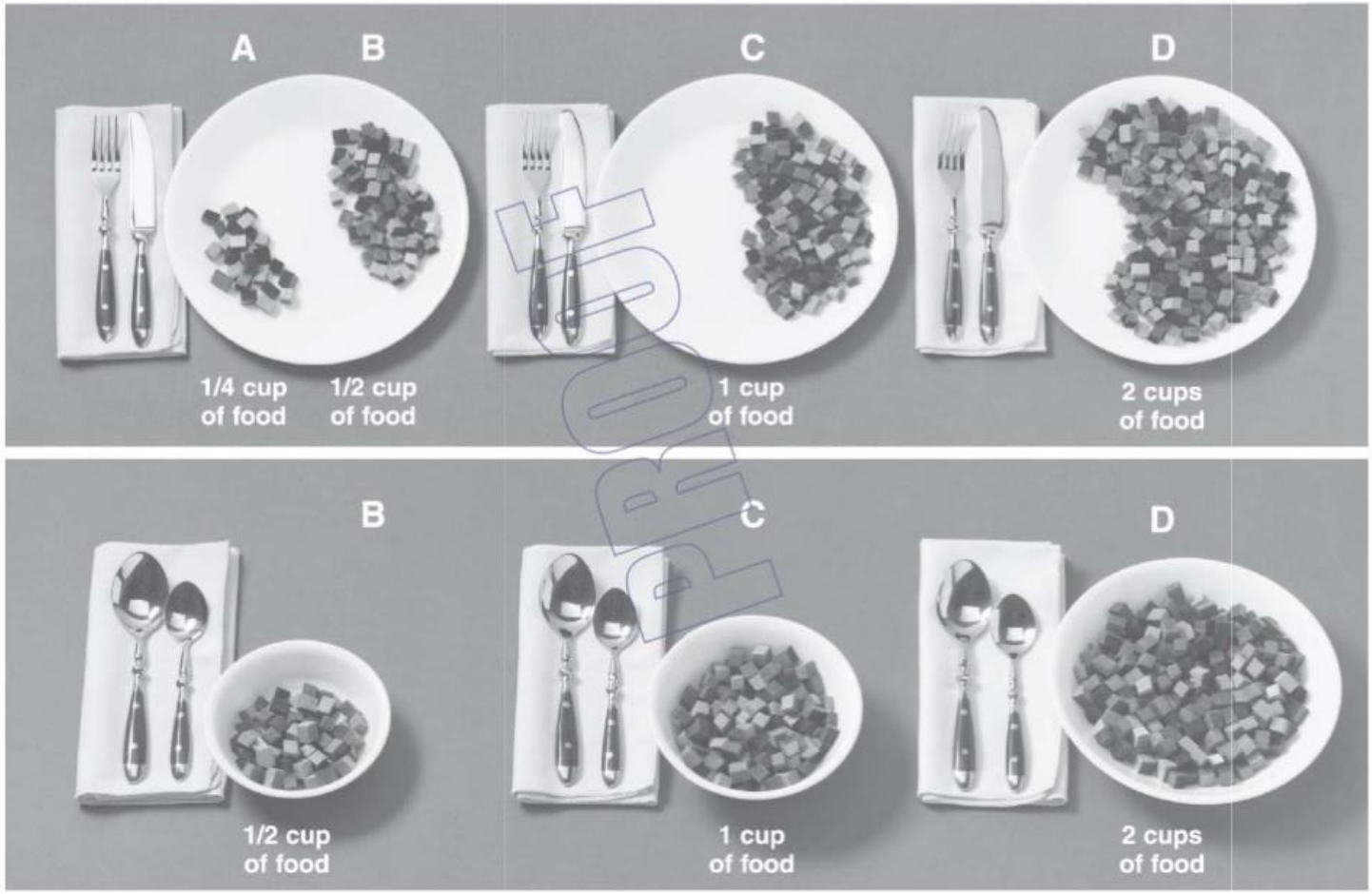
Carefully remove this page and use the portion size pictures on the back.

1/4" SPINE PERF

Portion Size Choices

Keep this in front of you while you are filling out The Food Questionnaire. You may use either the plates or the bowls to help you choose your usual portion size.

Choose A, B, C or D: **A** = 1/4 Cup of Food **B** = 1/2 Cup of Food **C** = 1 Cup of Food **D** = 2 Cups of Food



3.4 Sexual Maturation

Female Pubertal Stage

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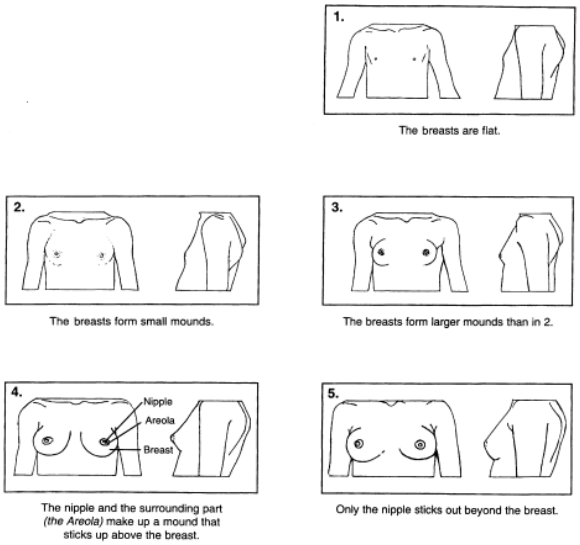
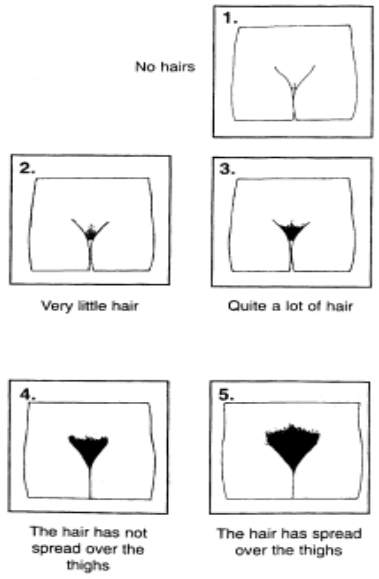
 # 1: PLEASE LOOK AT BREAST SIZE ONLY



 # 2: PLEASE LOOK AT PUBIC HAIR ONLY



Female Pubertal Stage

FACULTY OF APPLIED HEALTH SCIENCES - BROCK UNIVERSITY

NAME: _____

This scale is to assess your maturational level. For both columns, please choose the appropriate stage and write the corresponding number on this piece of paper.

Column #1: _____ (corresponding number)

Column #2: _____ (corresponding number)

Please answer the following questions:

1. Have you had your period?

   YES NO

2. If yes, how old were you when you had your first period? _____
3. How often do you get periods? (in days)_____
4. For how many days does your period last? _____

Appendix 4: Data Collection Sheet

ANTHROPOMETRIC MEASUREMENTS

DOB: _____

Height (cm):	Seated Height (cm):
Weight (kg):	Age from PVH (years):
BMI:	% Body Fat:
Hip Circumference (cm):	Waist Circumference (cm):
W:H Ratio:	Tanner Stage:

SPEED OF SOUND MEASUREMENTS

RADIUS:

TRIAL	SOS	T-SCORE	Z-SCORE
1			
2			
3			

TIME OF BLOOD DRAWS

Draw	1	2	3	4
Time				

Appendix 5: Data Not Appearing in Main Body of Thesis

5.1 Raw Data: Girls

Participant ID	Age (years)	Height (cm)	Seated Height (cm)	Age from peak height velocity	Weight (kg)	% BF	*FFM (kg)*	BMI	Waist Circumference (cm)	Hip Circumference (cm)	Waist to Hip Ratio
G02	8	129.6	66	-3	27.4	16.1	22.99	16.2	53.5	70.3	0.76
G03	8	127.8	67.5	-3.1	25.5	10.3	22.87	15.6	51.5	65	0.79
G04	8	132.5	74	-2.9	26.7	6.1	25.07	15	52	62	0.84
G05	12	155.2	76	0	41.9	22	32.68	17.4	59	76	0.78
G06	12	159	78	0.3	46.2	17.4	38.16	18.2	64.5	88.3	0.73
G07	10	138	68	-2.1	29.3	10.8	26.14	15.4	53	72	0.74
G08	12	146	63	-0.8	33.6	15.8	28.29	15.8	53	77	0.69
G09	9	144	74	-1.7	38.4	21.1	30.3	18.5	58	74.5	0.78
G10	9	144.6	70.5	-1.9	37.4	23.3	28.69	17.9	63.5	76.8	0.83
G11	13	159.7	80.5	1.5	49.7	16.7	41.4	19.4	63.2	83	0.76
G12	12	163.6	81.7	1.1	58.4	25.8	43.33	21.8	69	91	0.76

G13	12	149.3	73.7	-0.4	38.55			17.3			
G14	11	149	72	-0.5	38.55			17.4			
G15	11	141.3	67.9	-1	34.02			17			

Participant ID	Tanner Stage	Daily Energy Intake (kcal)	Daily Calcium Intake (mg)	METs per week	Sclerostin A (pmol/L)	Sclerostin B (pmol/L)	Sclerostin C (pmol/L)	Sclerostin D (pmol/L)
G02	1	1182.32	599.19	63	32.94	18.62	16.48	32.91
G03	1	1462.57	568.6	90	26.35	13.73	24.24	20.59
G04	1	1567.24	1058.5	78				17.78
G05	3	1092.03	835.97	91	16.48	9.22	11.03	13.65
G06	3	1218.22	509.31	88			29.04	32.38
G07	1	1305.2	771.87	177	26.84	28.71	30.51	31.99
G08	2	1190.77	647.55	62	29.88	28.29	26	35
G09	1	1044.93	463.26	89	32.82	32.43	32.69	32.82
G10	1	630.93	523.61	162	36.7	35.55	36.48	37.31
G11	2	1191.16	492.97	148	27.9	26.71	26.05	
G12	3	1231.61	403.8	66	42.77	43.6		34.28
G13	2	1303.23	393.96	109			37.2	
G14	1	495.44	312.14	94	36.34	34.3		
G15	1	1193.76	711.9	85	36.32	37.47	38.98	

Participant ID	DKK-1 A (pmol/L)	DKK-1 B (pmol/L)	DKK-1 C (pmol/L)	DKK-1 D (pmol/L)	OPG A (pmol/L)	OPG B (pmol/L)	OPG C (pmol/L)	OPG D (pmol/L)
G02	40.16	20.9	9.49	38.31	6.96	3.31	2.59	6.43
G03	42.98	24.74	38.24	28.82	5.82	3.44	4.65	3.18
G04				20.89				4.22
G05	36.84	25.56	25.19	30.19	3.82	2.57	2.85	3.74
G06		22.39	24.1	24.57		8.1	11.15	12
G07	22.15	22.46	23.79	24.1	6.5	6.93	7.43	7.35
G08	20.98	21.45	19.73	23.79	6.15	6.2	6.1	7.4
G09	29.02	29.48	28.78	26.91	7.75	7.8	7.7	7.3
G10	26.25	25.82	25.74	25.9	13.78	13.5	13.65	13.75
G11	23.91	26.73	22.5		17.1	18.68	17.3	
G12	31.36	33.48		25.74	16.6	17.1		14.15
G13			31.22				18.98	
G14	30.73	30.71			19.3	19.5		
G15	31.82	35.06	33.11		17.1	19.5	19.25	

Participant ID	RANKL A (pmol/L)	RANKL B (pmol/L)	RNAKLC (pmol/L)	RANKL D (pmol/L)	TGF-β1 A (ng/ml)	TGF-β1 B (ng/ml)	TGF-β1 C (ng/ml)	TGF-β1 D (ng/ml)
G02	2.37	1.74	1.74	2.03	2.23	1.75	2.01	2.16
G03		1.05		1.44	2.27	2.39	2.18	1.83
G04				1.19	0		0	2.08
G05	2.18	2.61	2.18	1.74	2.14	2.63	2.18	2.06
G06		1.6	1.6	2.45		0.67	0.68	1.05
G07	2.25	2.14	2.14	1.89	2.1	1.53	1.41	1.91
G08	2.39	2.39	0	2.39	2.53	1.8	1.86	1.74
G09	3.57	3.57	3.57	3.7	2.27	1.81	2.08	1.62
G10	2.8	2.02		2.5	1.41	1.12	1.21	1.09
G11					0		0.58	0
G12	2.34	2.6		2.64	0.89	1.43	0	0.76
G13			1.17		0		0.88	0
G14	1.95	1.64			1.02	1.05	0	0
G15		1.55	1.17		2.12	2.13	2.35	0

Participant ID	TGF-β2 A (pmol/L)	TGF-β2 B (pmol/L)	TGF-β2 C (Pmol/L)	TGF-β2 D (pmol/L)	TGF-β3 A (ng/ml)	TGF-β3 B (ng/ml)	TGF-β3 C (ng/ml)	TGF-β3 D (ng/ml)
G02	0.16	0.15	0.15	0.16	0.00488	0.00488	0.00528	0.00488
G03	0.17	0.16	0.15	0.14	0.00488	0.00528	0.00488	0.00408
G04				0.15				0.00488
G05	0.15	0.18	0.15	0.14	0.00448	0.00528	0.00488	0.00488
G06		0.08	0.09	0.13			0.00253	0.00253
G07	0.17	0.14	0.14	0.17	0.00408	0.00369	0.00360	0.00408
G08	0.18	0.15	0.15	0.15	0.00488	0.00448	0.00408	0.00448
G09	0.2	0.17	0.18	0.15	0.00269	0.00251	0.00287	0.003486
G10	0.14	0.13	0.12	0.12		0.00253		0.00253
G11	0.19	0.19	0.19		0.00473	0.00473	0.00533	
G12	0.19	0.19	0.14	0.19	0.00473	0.00533	0.003609	0.00533
G13			0.19				0.0059	
G14	0.19	0.19			0.00617	0.00533	0.002533	
G15	0.2	0.19	0.19		0.00533	0.0059	0.003609	

5.2 Raw Data: Adolescents

Participant ID	Age (years)	Height (cm)	Seated Height (cm)	Age from peak height velocity	Weight (kg)	% BF	FFM (kg)	BMI	Waist Circumference	Hip Circumference (cm)	Waist to Hip Ratio
T01	14	164.7	83	1.7	46.1	12.3	40.4297	16.9	61	78.5	0.777070064
T02	16	170	89	3.2	49	14.2	42.042	17	58	85.7	0.676779463
T03	15	166	84.7	2.9	69.3	28.7	49.4109	24.8	75.5	100	0.755
T04	14	160.1	82	1.6	56.2	24.5	42.431	21.9	71.4	89.5	0.797765363
T05	14	177	90	2.9	68.2	19.6	54.8328	21.9	70	95	0.736842105
T06	13	163.1	87	1.8	53.7	20.1	42.9063	20.2	67	91.5	0.732240437
T07	16	148.5	80	1.9	52.1	27.7	37.6683	23.6	67	91	0.736263736
T08	15	165	79	2.5	67.4	33.1	45.0906	22.6	76.2	89.5	0.851396648
T09	15	170.8	87.5	3.1	68.6	29.6	48.2944	23.5	77	94.5	0.814814815
T10	16	157	83		63.9	36.5	40.5765	25.9	84	106	0.79
T11	16	154	84		67	36.8	42.344	28.3	85	108	0.79

T14	16	163.7	85.5		57.3	21.1	45.2097	21.4	68	96	0.708333333

Participant ID	Tanner	METs per week	Daily Energy Intake (kcal)	Daily Calcium Intake (mg)	Sclerostin A (pmol/L)	Sclerostin B (pmol/L)	Sclerostin C (pmol/L)	Sclerostin D (pmol/L)
T01	5	64	1266.98	1211.6		30.58	33.264	29.084
T02		60	1393.27	597.12	32.78	33.176	32.384	34.54
T03	5	55	1231.57	562.5	29.348	29.348	25.608	25.608
T04	4	117	1637.32	1752.99	30.976	33.792	33.264	
T05	3	127	2500.28	1514.08	37.796	37.796	36.696	39.336
T06	3	88	1826.46	756.91	39.424	39.16	38.016	38.984
T07	4	116	2784.38	1206.96	34.98	34.848	35.156	33.814
T08	5	41	4105.75	2580.29				
T09	3	126	3909.57	2370.25	30.052	29.48	31.24	27.808
T10	5	40			29.568	29.304	30.976	27.72
T11	5	22			32.736	31.152	26.532	31.416
T14	4	130	1428.39	934.43	28.732	28.468	28.468	

Participant ID	DKK-1 A (pmol/L)	DKK-1 B (pmol/L)	DKK-1 C (pmol/L)	DKK-1 D (pmol/L)	OPG A (pmol/L)	OPG B (pmol/L)	OPG C (pmol/L)	OPG D (pmol/L)
T01		25.194	27.3	24.492		7.175	7.35	6.25
T02	17.94	18.096	17.94	18.096	6.35	6.25	6.5	6.15
T03	18.876	19.188	18.72	18.72	5.5	5.55	5.45	5.225
T04	23.868	25.584	25.038		7.45	11.825	9.125	
T05	17.16	17.472	17.16	16.848	5.9	6	6.1	6.05
T06	18.408	18.408	18.564	18.408	6.8	6.9	6.9	7
T07	18.876	18.954	18.564	19.11	6	5.8	6.05	6
T08	27.456				6.75			
T09	28.08	28.86	28.548	27.924	6.95	7	7	6.75
T10	26.13	26.13	23.673	25.662	11.3	11.15	8.15	10.325
T11	25.272	24.336	25.35	22.893	11.625	8.725	9.95	6.675
T14	31.434	31.8435	32.5065		16.725	17.1	18	

Participant ID	RANKL A (pmol/L)	RANKL B (pmol/L)	RANKL C (pmol/L)	RANKL D (pmol/L)	TGF-β1 A (ng/ml)	TGF-β1 B (ng/ml)	TGF-β1 C (ng/ml)	TGF-β1 D (ng/ml)
T01		1.4005	1.194	1.194		1.682	1.4575	0.9455
T02	1.194	1.06	1.06	1.06	1.506	1.671	1.501	1.391
T03	2.198	1.6125	2.3485	1.902	1.629	1.935	2.008	2.023
T04	3.813	3.846	3.657		2.572	2.0215	1.947	
T05	2.5	1.4705	1.194	1.756	1.5855	1.836	1.703	1.1525
T06		1.6125	1.2625		1.0215	0.8855	1.161	1.015
T07		1.2625		1.045	1.218	1.303	1.138	0.9965
T08	1.839				0.9305			
T09	2.391	2.391	1.839	2.559	0.992	0.99	2.241	0.843
T10	3.625	3.675	2.725	3.625	0.9655	0.692	0.7715	1.027
T11	2.8	2.451	3.75	2.55	0.3665	0.52	0.677	0.712
T14	1.8085	1.3165	1.1735		0.41617	1.04042	1.53985	1.13335

Participant ID	TGF-β2 A (ng/ml)	TGF-β2 B (ng/ml)	TGF-β2 C (ng/ml)	TGF-β2 D (ng/ml)	TGF-β3 A (ng/ml)	TGF-β3 B (ng/ml)	TGF-β3 C (ng/ml)	TGF-β3 D (ng/ml)
T01		0.1135	0.109	0.1065		0.00253		
T02	0.139	0.152	0.137	0.133	0.00235	0.00278	0.00235	0.00251
T03	0.13	0.15	0.15	0.153	0.00235	0.00235	0.00251	0.00234
T04	0.192	0.153	0.147	0	0.00251	0.00287		
T05	0.1255	0.164	0.159	0.131	0.00234	0.00234	0.00253	
T06	0.118	0.11	0.133	0.126	0.00253	0.00253	0.00253	
T07	0.125	0.1245	0.1475	0.1045	0.00253	0.00358	0.00253	
T08	0.10081	0.191	0.19	0.191				
T09	0.09469	0.09631	0.1975	0.08449	0.00253	0.00253	0.00253	
T10	0.085015	0.063	0.08441	0.08739	0.00167	0.00167	0.00212	0.00156
T11	0.05112	0.04912	0.04661	0.06935	0.00145	0.00145	0.00145	0.00167
T14					0.00539	0.0059	0.0059	